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UNIVERZITA KARLOVA V PRAZE
PŘÍRODOVĚDECKÁ FAKULTA

Studijní program: Biologie
Studijní obor: Genetika, molekulární biologie a virologie

Bc. Jakub Soukup

Optimalizace metod pro studium časných fází životního cyklu myšího polyomaviru

Optimization of methods for analysis of early steps of mouse polyomavirus life cycle

Diplomová práce

Školitel: RNDr. Hana Španielová, Ph.D.

Praha, 2015

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 14. 8. 2015

Podpis:

Jakub Soukup

I would like to express my gratitude to my supervisor **RNDr. Hana Španielová, PhD**. for excellent guidance during my work in laboratory. I appreciate her invaluable advice, help and ideas.

Also, I would like to thank head of our laboratory **Doc. RNDr. Jitka Forstová, CSc**. for the opportunity to work in her group.

I would like to thank all my colleagues for their support, ideas and for creating a good working environment. Especially, I want to thank **Mgr. Jiřina Suchanová** for her advices, **Mgr. Martin Fraiberk** and **Mgr. Vojtěch Žíla, PhD**. for help with electron microscopy, **Mgr. Boris Ryabchenko** and **RNDr. Lenka Horníková, PhD**. for help with confocal microscopy.

Last but not least, I want to thank **Vlasta Sakařová** and **Ivana Poívková** for excellent technical support.

This work was kindly supported by the Grant Agency of the Czech Republic (Project P302/13-26115S), by the Ministry of Education, Youth and Sports of the Czech Republic (Project SVV-SVV-2013-267205) and by the Grant Agency of Charles University (Project GAUK/913613)

Abstract

Mouse polyomavirus is a type species of *Polyomaviridae* family and serves as model for studying viral infection of human pathogenic polyomaviruses. Minor proteins of viral capsid have been found to be necessary for effective initiation of infection. In order to study their role in the early steps of infection we utilized the novel Cre-LoxP system for production of the viral mutant lacking both minor proteins. Virus produced this way was compared with virus produced by standard method and we found that both systems facilitate production of mutant virus with the comparable quality and quantity. The mutant virus contained reduced amount of viral DNA and formed virions with impaired stability. For further studies of intracellular virion trafficking we prepared virions with genomes modified by thymidine analogues 5-bromo-2´-deoxyuridine (BrdU) and 5-Ethynyl-2´-deoxyuridine (EdU) and optimized the methods for analogue detection. The viral genome become accessible for detection 4 hours post infection. For ultramicroscopic analysis of translocation of virus to the nucleus we used freeze substitution. All this methods will be utilized for detailed study of distinct steps in viral infection.

Key words: Mouse polyomavirus, minor proteins, mutant virus, Cre recombinase, BrdU, EdU, Click Chemistry

Abstrakt

Myší polyomavirus je typickým zástupcem čeledi *Polyomaviridae* a slouží jako model pro studium virové infekce lidskými patogenními polyomaviry. Minoritní proteiny virové kapsidy se ukázaly jako nezbytné pro efektivní zahájení infekce. Za účelem studia jejich role v časných fázích infekce jsme využili nový Cre-LoxP systém produkce virové mutanty postrádající minoritní proteiny. Virus produkovaný touto cestou byl porovnán s virem produkovaným standardní metodou a zjistili jsme, že oba systémy umožňují produkci mutantního viru se srovnatelnou kvalitou a kvantitou. Mutantní virus obsahoval snížené množství virové DNA a tvořil kapsidy se zhoršenou stabilitou. Pro další studium vnitrobuněčného transportu virionů jsme připravili viriony s genomem modifikovaným analogy tymidinu 5-bromo-2´-deoxyuridin (BrdU) a 5-Ethynyl-2´-deoxyuridin (EdU) a optimalizovali metody detekce analogu. Virový genom se zpřístupní detekci 4 hodiny po infekci. Pro ultramikroskopickou analýzu dopravy viru do jádra jsme použili mrazovou substituci. Všechny tyto metody budou použity pro detailní studium odlišných kroků virové infekce.

Klíčová slova: Myší polyomavius, minoritní proteiny, mutantní virus, Cre rekombináza, BrdU, EdU, Click Chemistry

Content

Abbreviations

AGT	O^6-alkylguanin-DNA-alkyltransferase
AP	Acceptor Peptide
BG	O^6-benzylguanine
BrdU	5-bromo-2´-deoxyuridine
DiD	1,1´-dioctadecyl-3,3,3´,3´-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt
DIG	Digoxigenin
EdU	5-Ethynyl-2´-deoxyuridine
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate (excitation filter - 475 to 490 nm)
HAU	Hemagglutination Unit
HBV	Hepatitis Beta Virus
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidase
HSV	Herpes Simplex Virus
MCPyV	Merkel Cell Carcinoma Polyomavirus
MOI	Multiplicity of Infection
MPyV	Mouse Polyomavirus
NLS	Nuclear Localization Signal
PARP	Poly ADP Ribose Polymerase
PDI	Protein Disulfide Isomerase
QD(s)	Quantum Dot(s)
RCNMV	Red Clover Necrotic Mosaic Virus
SNAP-tag	Self-Labelling Protein Tag
SV40	Simian Vacuolating virus 40
THPTA	Tris(3-hydroxypropyltriazolylmethyl)-amine
TRITC	Tetramethylrhodamine isothiocyanate (excitation filter - 545 to 565 nm)
VLP(s)	Virus like particle(s)
VSV	Vesicular Stomatitis Virus

1. Introduction

Polyomaviruses are tumorigenic non-enveloped DNA viruses widely spread among the human population and establish latent asymptomatic infections unless the patient is not immunodeficient. Merkel Cell Carcinoma polyomavirus is the only one which causes aggressive carcinoma in patients. Polyomaviruses are able to form virus like particles (VLPs) which are tested for vaccine development, drug delivery or gene therapy. For efficient utilization of these applications we need to thoroughly examine the life cycle of polyomaviruses and especially the early steps of infection. The early steps of MPyV infection are not well characterized yet and especially, the role of minor proteins - VP2 and VP3. A good instrument for characterization of these proteins is viral mutants without VP2 and VP3 (VP1_only virus). Virus can form the capsid without minor proteins but it lacks infectivity.

In the first part of my thesis I am adapting Cre-LoxP system of virus production to VP1_only virus production and comparing it with standard method of VP1_only virus production. The Cre-LoxP system is simpler and faster than standard type. Utilization of this system in wild-type virus provided similar yields of infectious viral progeny as the standard method (Hron et al., 2013). Furthermore, we analysed the VP1_only mutant.

The second part of my thesis is dedicated to adaptation of novel method of genome detection to our research. When we are studying the early steps of MPyV infection we detect viral proteins. But the proteins are not the infectious part, the genome is infectious. MPyV trafficking to the nucleus is not properly examined and the novel method would allow us to track viral genome in cells instead of proteins. This approach is especially useful for therapeutic gene packed in the capsid. We have incorporated thymidine analogues into the viral genome which can be labelled with antibody or with Click Chemistry. This novel approach could contribute to the elucidation of MPyV trafficking to the cell nucleus and thus could help to design more efficient utilization of MPyV for therapeutic applications.

Third part is dedicated to the utilization of high pressure freezing and freeze substitution in electron microscopic analysis of infected cells to study the MPyV entry to the nucleus.

All parts together are directed to elucidation of MPyV life cycle. Labelled viral genomes can be tracked with fluorescent and electron microscope. Also we can label the genome inside the VP1_only virus and thus perform comparative analysis of VP1_only virus and wild-type virus. With labelled genome in non-infectious virus we can elucidate the pathways which do not lead to infection.

2. Literature review

2.1 Polyomaviruses

Polyomaviruses belong to *Polyomaviridea* family with the broad range of hosts in vertebrates, including humans. In 2010 the single *Polyomavirus* genus was divided into 3 genera - *Orthopolyomavirus*, *Wukipolyomavirus* and *Avipolyomavirus* where the first and second group are formed for mammalian viruses and the third group is formed for avian viruses *(Johne et al., 2011)*. To the date is known 15 polyomavirus species which infect human cells - the most known are BK polyomavirus, JC polyomavirus and Merkel Cell Carcinoma Polyomavirus (Mishra et al., 2014; Rinaldo and Hirsch, 2013). It was discovery of Merkel Cell Carcinoma Polyomavirus which caused boom of polyomavirus research (Feng et al., 2008).

There are 2 main virus models for polyomavirus study: The Mouse polyomavirus (MPyV) discovered as first polyomavirus in leukemic mouse (Gross, 1953) and Simian Vacuolating virus 40 (SV40) discovered in poliovirus vaccine (Sweet and Hilleman, 1960). The focus of our research is in MPyV life cycle, specifically in early steps of infection. The Second area of our research is utilization of Mouse polyomavirus particles.

Polyomavirures are small viruses with the icosahedral capsid with diameter around 45 nm (Fig. 2.1 A). Capsid of MPyV is formed from 3 structural proteins called viral protein (VP) 1, VP2 and VP3. VP1 is the main structural protein of the capsid with size of 45 kDa and forms pentamer structure which is the basic structural component of virus capsid. N-terminus of VP1 is oriented into the central cavity and it is responsible for DNA binding. It also contains nuclear localization signal. C-terminus binds other VP1 molecules and it is responsible for forming VP1 pentamer. VP1 also mediate virion binding to the cellular receptor. Each pentamer of VP1 contain one minor protein VP2 or VP3 in central cavity. VP2 or VP3 is inserted in VP1 with C-terminus and it is linked to the VP1 monomers via hydrophobic interactions. VP3 is smaller protein than VP2 with size of 23 kDa. Entire sequence of VP3 is contained in C-terminus of larger VP2 protein and size of VP2 protein is 35 kDa. The capsid covers minichromosome with the viral genome which is encoded in circular double-stranded DNA with length of 5,307 base pairs (Fig. 2.1 B). Minichromosome is also composed of cellular histones H2A, H2B, H3, H4 in form of octamers. The viral genome is organized into three main regions: early, late and regulatory region. Regulatory region is consisting of origin of replication (Ori), enhancer of transcription, late and early region promoters. In the infection, first is expressed early region and expression continues to the late stages of infection. Early region encodes 3 regulatory

proteins in MPyV: large T-antigen, middle T-antigen and small-T-antigen. These proteins are synthesized from common pre-mRNA which undergoes alternative splicing. Late region of MPyV genome encodes three proteins: VP1, VP2 and VP3. As in the early region, transcription of late region synthesizes pre-mRNA molecule which undergoes alternative splicing (Knipe and Howley, 2013).

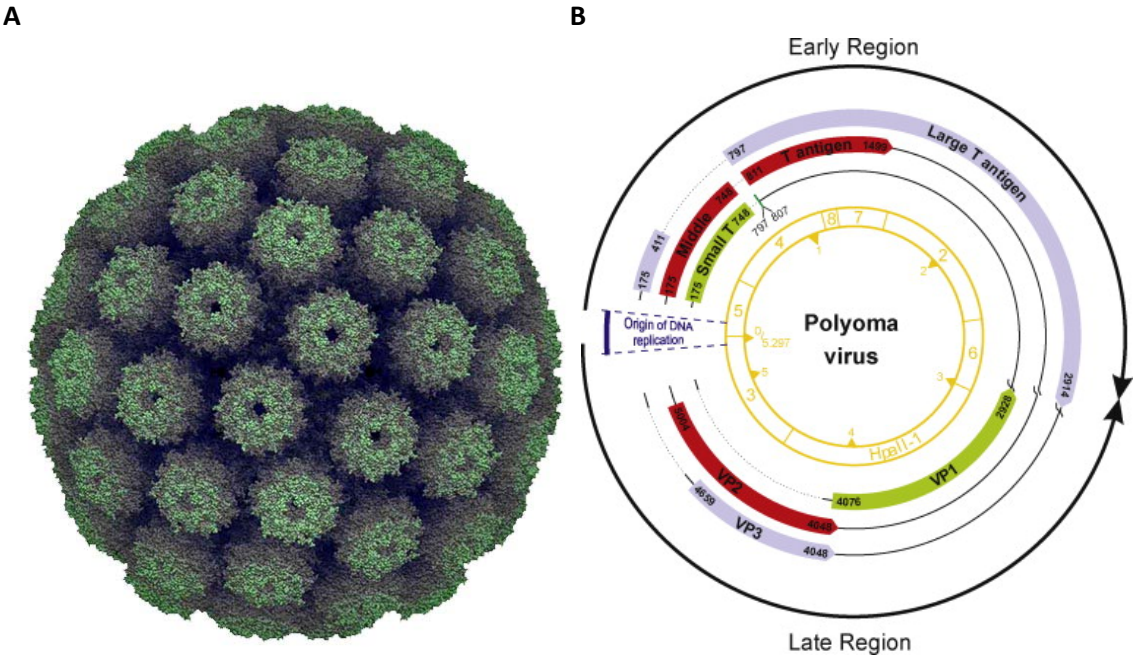


Figure 2.1: **A:** The structure of Mouse polyomavirus capsid. VP1 pentamers are illustrated on picture. Adapted from Stehle and Harrison (1996). **B:** Genome organization of polyomavirus. Early and late transcriptions are symbolized by black arrows. Adapted from Atkin et al. (2009).

2.1.1 Role of minor proteins in life cycle of mouse polyomavirus

Minor capsid proteins are important for successful infection of cells and perhaps the encapsidation of viral genome. Particles without minor proteins are defective in infection and it was shown by many independent groups working on MPyV (Mannova et al., 2002; Sahli et al., 1993), SV40 (Nakanishi et al., 2007; Nakanishi et al., 2006)or JC (Gasparovic et al., 2006). In polyomaviruses which consist only of VP1 and VP2 (MCPyV clade) is situation different. The importance of VP2 is connected to the cells which are infected. In one type of the cell the VP2 is crucial to infection in the other type VP2 is dispensable (Schowalter and Buck, 2013).

The life cycle of Mouse polyomavirus (Fig. 2.2) starts with the attachment to the membrane. Minor proteins are inside the capsids so it is impossible for them to interfere with attachment or virion binding. The only possible way is that their presence stericaly affect the

structure of the virion. Even a small change in structure could alter the final morphology and thus receptor binding for example. This phenomenon is reported in rhinoviruses (Emini et al., 1983; Pevear et al., 1989). This theory is supported with SV40 studies. VP2 of SV40 considerably increases its internalization (Daniels et al., 2006; Enomoto et al., 2011). MPyV recognize cellular surface receptors GD1a and GT1b (Tsai et al., 2003). After attachment to the cellular membrane, virus enters in the cell and is localized in early endosome (Mackay and Consigli, 1976; Mannova and Forstova, 2003; Richterova et al., 2001). Unlike other viruses, polyomaviruses probably do not exit acidic endosomes via fusion of membranes (enveloped viruses) or penetration of vesicle (non-enveloped virus). Instead, polyomaviruses are translocated into ER in endocytic vesicles. Pathways which include recycling endosome, late endsome and endolysozomal complex were published for MPyV (Liebl et al., 2006; Mannova and Forstova, 2003). It is shown that MPyV requires short exposure to the acidic pH of late endosomes for successful infection (Liebl et al., 2006). It is still not clear, how do polyomaviruses get to ER. One study shows that MPyV bypass Golgi complex but other studies (Mannova and Forstova, 2003) shows viral sensitivity to drugs inhibiting traffic inside Golgi complex and traffic from Golgi complex to ER. Drugs inhibiting traffic from Golgi complex to ER (Brefeldin A) are not specific and inhibit all retrograde transport (Gilbert and Benjamin, 2004) and thus this drugs could modify the delivery indirectly. It seems that in these steps of infection, minor protein plays no role but once MPyV or SV40 is translocated in ER it undergoes partial disassembly and genome exposure in ER due to effect of lumenal enzymes (Magnuson et al., 2005; Walczak and Tsai, 2011). When the SV40 capsid is partially disassembled minor proteins are exposed on the surface of the virion (Kuksin and Norkin, 2012). There are two possible ways of delivery from ER to the nucleus. First is directly from ER to the nucleus by penetration through nuclear membrane (Butin-Israeli et al., 2011). The translocation though nuclear membrane would be possible if virus has a tool for penetration of membranes. So-called viroporin activity has been shown for VP3 protein of SV40 (Daniels et al., 2006). The second possible way involves transport to the cytoplasm and import as a cargo through nuclear pore complex (Nakanishi et al., 2002; Yamada and Kasamatsu, 1993).

Once virus gets to the nucleus, the transcription of early region starts (Cogen, 1978). It has not been reported any function of minor proteins in the transcription or replication of the genome. But once VP2 and VP3 are synthesized in the cytoplasm they could mediate translocation of VP1 into the nucleus (Lenka Horníková, unpublished observations). Role of minor proteins in encapsidation of polyomavirus was not reported. There could be a role of minor proteins in release of the virions. The minor proteins have a role in apoptotic, necrotic or possible necroptotic processes. SV40 VP3 and VP4 protein were reported as a trigger for

lytic processes (Daniels et al., 2006). Also, these proteins were reported to bind PARP and stimulate its enzymatic activity and thus mediate necrosis (Gordon-Shaag et al., 2003). The study on SV40 is supported by study on MPyV. MPyV without minor proteins do not induce cell lysis and thus VP2 or VP3 is the inducer of apoptosis in MPyV (Huerfano et al., 2010).

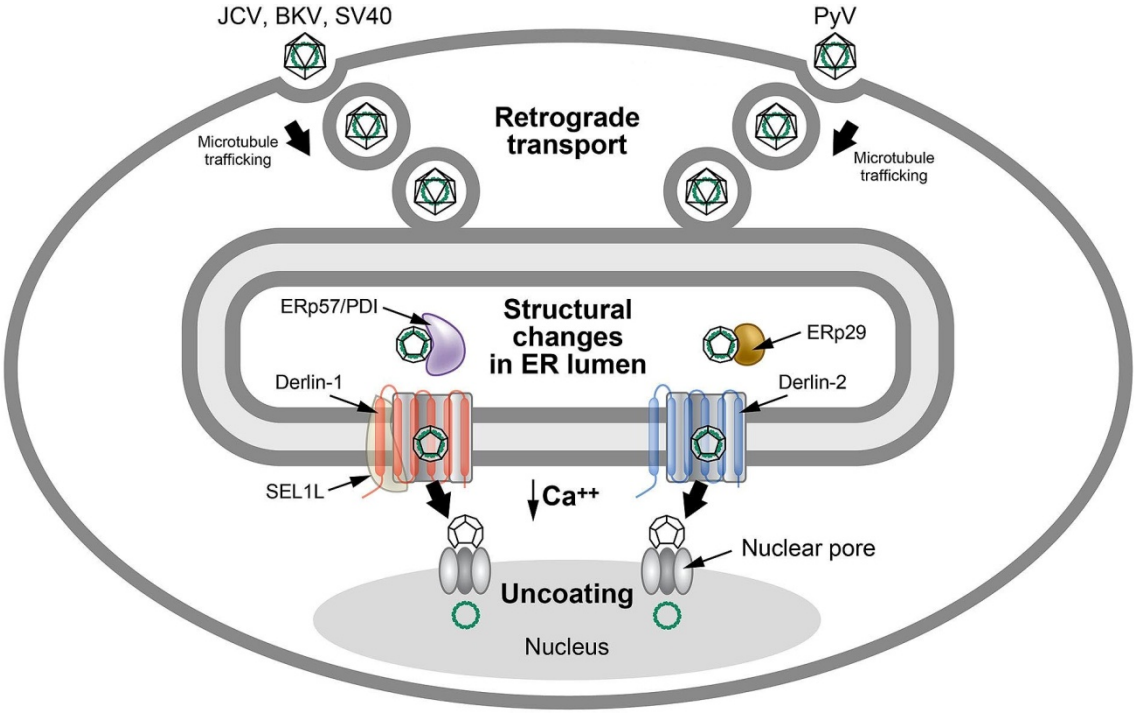


Figure 2.2: The schematic overview of the polyomavirus life cycle. Virus is attached to the membrane and enters the cell via receptor mediated endocytosis. Virus travels to ER where is viral particle modified. Modified virus enters the nucleus directly from ER through nuclear envelope (this way is not illustrated on scheme) or virus enters the cytoplasm and enters to nucleus via nuclear pore complex. Adapted from Byun et al. (2014).

Polyomaviruses differ in the production of minor proteins. For example SV40 is coding 3 minor proteins (Daniels et al., 2007) and on the other hand MCPyV codes only 1 minor protein (Schowalter and Buck, 2013).Mutations in minor proteins can alter the fate of the virus.

2.1.2 Structure of minor proteins

VP2 is composed from 319 aminoacids with protein mass 35 kDa. VP2 has a unique N - terminal sequence compared to VP3 (residue 1-115). VP2 C-terminus folds into α-helix structure and N-terminus is not folded in complex with VP1. Group of amino acids responsible

for binding to VP1 pentamer is localized in position 266 - 301. This binding has hydrophobic character (Chen et al., 1998)(Fig. 2.3)

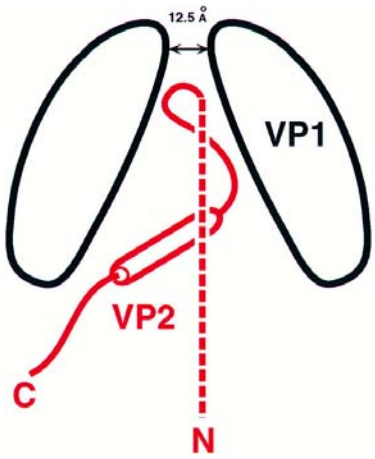


Figure 2.3: Schematic structure of VP1 pentamer (only two monomers are shown in side view) with VP2 (full and dashed line) or VP3 (only full line) in its cavity (Chen et al., 1998).

In VP2 is known one hydrophobic post-translational modification. It is located in N-terminus where glycine in VP2 is modified by myristic acid (Streuli and Griffin, 1987). No other post-translation modification which could play a role in membrane localization (modification with hydrophobic group) has been discovered so far but minor proteins contain other post-translational modification.

The most known post-translation modification are in minor protein of BK. Fang et al. (2010) found many modifications of VP2 and VP3 with smaller chemical groups. Despite that C-terminus of VP2 has identical sequence with VP3, there were found different modification for the same amino acids. The Ser-129 in VP3 (Ser-248 in VP2) is the only amino acid which is phosphorylated in VP3 of BK virus. The C-terminal residues (214-318 in VP2, 95-199 in VP3) are hydrophobically bonded to VP1 and founded phosphorylation in this region could play a role in binding to VP1. Phosphorylation has been also studied in SV 40 in 1970´s but biological function of these modification on specific amino acid residues remain unknown (Ponder et al., 1977; Tan and Sokol, 1972). The most important modification seems to be the N-terminal myristilation of VP2 and the loss of myristic acid result on result in lower infection.

VP2 and VP3 contain nuclear localization signal (NLS) in 11 terminal amino acids of common C-terminus (Chang et al., 1992). Even though MPyV minor proteins contain NLS, the transport to the nucleus is not effective. Minor proteins are mainly transported into nucleus in complex with VP1 (VP1 posses its own NLS) in insect cell (Forstova et al., 1993). On the other hand, SV 40 minor proteins are capable of nuclear transport on their own (Clever and Kasamatsu, 1991) and NLS of other structural protein can complement the nuclear localization

(Ishii et al., 1994). Prediction of NLS in MCPyV minor protein suggest that MCPyV encode NLS in VP1 N-terminus but not within VP2 (Schowalter and Buck, 2013). For efficient translocation of JC VP1 in the nucleus are needed both minor proteins. The mutation in VP2 or VP3 of JC reduces the localization of VP1 to the nucleus (Gasparovic et al., 2006)

2.1.3 Mutations in minor capsid proteins

Mutants are very important in research of almost every biological function. In minor proteins of polyomaviruses are used deletion mutations or mutation of myristyl residue in VP2 N-terminus. The deletion mutant phenotype differs greatly in polyomavirus family. The myristyl group on N-terminus of polyomaviral VP2 was discovered in late 1980´s (Streuli and Griffin, 1987). The myristylation is highly conserved in polyomaviruses and may account to the different localization of VP2 and VP3 (Forstova et al., 1993; Stamatos et al., 1987).

2.1.3.1 MPyV

Krauzewicz and her colleagues (1990) made 2 mutants of MPYV VP2 in glycine residue which is myristylated. They have substituted glycine for valine or glutamic acid. For subsequent studies was used the mutant with glutamic acid. When glycine was substituted for glutamic acid efficiency of infection was severely dropped to the one tenth of the wild-type virus. Authors hypothesize that myristylation could alter the stability of virions or altered morphology of virions. VP2 is not on the surface of virions but even a small change in structure could alter the final morphology and thus receptor binding for example. This phenomenon is reported in rhinoviruses (Emini et al., 1983; Pevear et al., 1989). The mutated virus has also higher proportion of disrupted particles. SV40 mutant genome isolates from cells appeared similar to the wild type. The region of mutation is not reported to be involved in the replication. The data suggested that myristylated VP2 is important for early stages of infections but it is not totally necessary for infection.

Mannová et al. (2002) analyzed myristylation sites of MPyV VP2 N-terminus. The glycine amino acid responsible for myristylation was mutated to the histidine (H), glutamine (Q) or glutamic acid (E). Mutants without myristic acid do not exhibit cytopathic effect. The infectivity decrease in following manner: wt > Q > E > H. The Q mutation infectivity is similar to the alanine mutation in (Sahli et al., 1993) but E and Q mutants do not exhibit defect described for alanine mutant. Myristylation of MPyV VP2 is not obligatory for infection but the infection

is severely reduced. It seems that VP2 myristilation has role of fine-tuning of VP2 function and slightly modifying virus.

Artificial capsids produced in baculovirus system composed only of VP1 protein were compared to wild-type virus. There has been no difference in trafficking of VP1 capsids and wild-type virus. The difference in delivery could be caused by difference in structural changes and surface conformation in trafficking. Secondly, VP1 only pseudocapsids could be unable to uncoat DNA properly (Richterova et al., 2001).

Charbonneau and her colleagues (2000) created 3 mutants in minor capsid proteins of MPyV by introducing stop codons in various position (substitution in positions 4684; 4613 or 4078 of late region). One mutant should produce polypeptides of length 129 amino acids (truncated VP2) and 14 amino acids (truncated VP3) but no VP2 or VP3 was detected. The mutant in position 4684 is missing VP2 completely. In the last mutant 4078 which should have 11 amino acid missing in C-terminus of VP2 and VP3, no VP3 or VP2 is detected. Also, the authors of this study did not detect VP1 which was not affected by substitutions and thus this mutant probably quickly disappears from transfected cells. The substitution at position 4684 (truncated minor proteins) had positive effect on DNA accumulation. The VP2 was shown to stimulate recombination.

2.1.3.2 SV40

Cole et al. (1977) designed 3 groups of mutants located throughout the SV40 genome for characterization of SV40. They have found that mutant without VP3 is not viable. In SV40 VP1 is responsible for membrane attachment and entry but virus without VP2 and VP3 SV40 probably not infectious. A VP3 deletion mutant and VP2 /3 deletion mutant used in this study showed a similar phenotype to the study of Li et al., (Li et al., 2003). All mutants are able to attach to the membrane and entry the cell but prematurely disassemble. It seems that the deletion mutants can modify integrity of the virion (Nakanishi et al., 2007).

Later, the SV40 virions without VP2 was shown to remain infective as long as they have VP3 (Gharakhanian et al., 2003) which is in conflict with previous study. The study of Cole et al., (1977) was not done on selective mutation of VP2 and VP3 and thus mutations could abolish the infection some other way. VP3 also triggers cell death by stimulation of PARP (Poly ADP Ribose Polymerase) which is activated by VP1 pentamer - VP3 capsid building blocks but DNA replication or late protein expression are not affected. This suggest that SV40 life cycle is not affected with poly(ADP)ribosylation by PARP (Gordon-Shaag et al., 2003). As mentioned above, VP1 can mediate translocation of VP3 with deletion in NLS to the nucleus. Based on the

homology model for VP1 and VP3 were identified sites responsible for binding of VP3 to VP1. If the site responsible for binding to VP1 in VP3 (2 sites - residues 155 - 190 and residues 222-234 both in common region with VP2) was mutated the viability of SV 40 was dropped. If the VP1 was mutated (point mutation in V243E or L245E or both) in binding site for VP3 the viability of SV40 was abolished (Nakanishi et al., 2006).

VP1 of SV40 was found to regulate VP2 and VP3 membrane localization. VP2 significantly enhance the virion-cell binding capacity. VP2 and VP3 are post-translational integrated into ER membrane in absence of VP1. This supports the theory that minor proteins act as viroporins after the virion disassembly. The VP3 can oligomerize itself. SV40 particles without VP2 showed 3-fold lower cell association. VP1 is produced 12 hours prior to VP2 and VP3 production. When minor proteins are produced, they are bind to the VP1 pentamers to prevent their binding into ER membrane (Daniels et al., 2006).

2.1.3.3 BK polyomavirus

No mutation of myristylation site or VP2 and VP3 was reported. Pseudovirions of BK polyomavirus formed only of VP1 had dramatically lower transduction efficiency than pseudovirions formed of all 3 viral proteins. The phosphorylation sites of BK polyomavirus were mutated in VP2. Modified serines were substituted for alanines. The mutation at position 254 is crucial for production of LT antigen and VP1. Authors believe that this post-translational modification is important for replication. The other 2 serines on position 223 and 248 had similar but mild effect on production of VP1 and LT (Chen et al., 2011).

2.1.3.4 JC polyomavirus

To investigate minor proteins of JC virus a directed mutagenesis was performed to mutate start codons of minor proteins or to mutate myristylation site in VP2. The myristylated glycine was replaced by alanine, glutamine, glutamic acid or histidine. With deletion mutants investigators found that JC virus needs at least one minor protein for efficient nuclear localization of VP1. As other polyomaviruses, minor proteins and VP2 myristilation are needed for infectious progeny production and minor proteins cannot substitute for each other. If the myristylated glycine was substituted for glutamic acid, the progeny showed altered morphology. Capsids of without myristylated VP2 were not as compact and regular as capsids with myristylated VP2(Gasparovic et al., 2006). JC virus capsids formed only with VP1 also do not protect its DNA genome from DNase degradation (Wang et al., 2004)

2.1.3.5 MCPyV

Observation of MCPyV infection revealed that MCPyV do not exhibit lytic or cytopathic effects on cells. Lytic effects of other polyomaviruses are caused by VP3 (VP4) protein. In MCPyV is VP3 missing. Schowalter and Buck (2013) speculate that MCPyV the VP1 protein, which is larger than other polyomaviral VP1 protein, could partially supplement VP3 protein function. Virus with deleted VP2 protein does not alter trafficking, binding of particles on cellular surfaces or packaging but infectivity was reduced severely. The same phenomenon occurred in virus with mutated myristilation site. Myristylation mutant observation suggested that myristyl group of VP2 is important during entry of MCPyV and possible penetration of cellular membranes. Important in this study is selected cell line. Mutants (VP2 deletion mutant or mutant in myristylation site) infectivity is reduced in certain cell lines but not in others.

2.3 Virus tracking in living cells

From the attachment to the membrane, a virus interacts with many components of its host. The importance of microscopic methods is increasing with the advance of tracking methods in the living cells. Cells are usually infected with multiple virions and in the most cases, each single virus undergoes different path. The static observation of fixed samples in light or electron microscope lacks the dynamic of infection and cannot provide detailed information. The single particle tracking allows unravelling the dynamics of infection and mechanism in each step of infection with single virus and various paths can be observed in the real-time. Real-time imaging is done with fluorescence microscope and requires labelling of the virus. Virus can be labelled with two methods: The fusion of viral proteins with fluorescence protein or direct labelling with fluorescence compound (Griffin et al., 1998; Shaner et al., 2005). Methods of virus labelling without lowering infectivity greatly improved over the past decade. Invention of super-resolution microscopy enhances possibilities of single particle tracking. On this level even interactions on molecular level can be observed (Godin et al., 2014).

Virus can be labelled in many ways depending on its structure. Virus envelope, capsid, non-structural proteins or genome can be labelled in different ways. There is one main obstacle in labelling virus - the infectivity. Many approaches to label virus end up in non-

infectious particles. Critical is the choice of the dye. A good dye should provide enough signal to detect single particle and it should withstand long exposures to light during imaging. Complex studies should label each part of virus - genome, capsid and envelope (in enveloped viruses) to provide information about all steps of infection.

2.3.1 Fluorescent protein tag

Viruses can be labelled with fluorescent protein tags such as GFP. These methods require recombinant proteins fused with fluorescent protein. Genetic recombinations provide labelling of exact position and strong signal but the fusion with fluorescent protein is tricky. The fusion site in the wrong place could lead to non-fluorescent protein due to immature chromospheres, misfolding or self-quenching in small viruses (Baumgaertel et al., 2012). Labelling with fluorescent proteins is quite common. There are works that should be highlighted. Authors of these works are fusing fluorescent protein with proteins in different parts of the virus. For example, foamy viruses (spumaviruses) have been labelled in two parts - the capsid proteins and envelope proteins with eGFP and mCherry (Stirnnagel et al., 2012). Even triple labelling was achieved in HSV-1. Envelope, tegument and capsid were successfully fused with fluorescent proteins (RFP, CFP, YFP). With such complex labelling viral infection and traffic of each part can be studied (de Oliveira et al., 2008). Genetic recombination of viral proteins with fluorescence protein is a powerful tool mainly in large viruses where the relatively large fluorescent proteins do not affect the infectivity of the virus. A discovery of photoswitchable fluorescent protein (cerFP505) started using of these fluorescent proteins in super-resolution microscopy based on photoswitching of fluorophores (PALM, FPALM, STORM, dSTORM) (Vogt et al., 2008).

The fusion of viral proteins with relatively large fluorescence proteins such as GFP may lower or abolish the infectivity or prevent assembly of the virus. In 1998 new labelling system was discovered which uses biarsenical-tetracystein technology (Griffin et al., 1998). Small peptide sequence (-CCXXCC-) is inserted into the protein and then biarsenical compound is bound to this tetracysteine structure. These biarsenical compounds (derivates of fluorescein - FlAsH-EDT2 and resorufin - ReAsH-EDT2) are membrane permeable and they are not fluorescent until covalently attached to tetracystein structure. This system of labelling has been shown in many types of viruses (E.g. Vesicular stomatitis virus, HIV-1, Ebola virus, Influenza, Bluetongue virus) (Du et al., 2014; Li et al., 2010; Mire et al., 2009; Panchal et al., 2003; Pereira et al., 2011). A similar method to tetracystein tag called SNAP-tag (self-labelling

protein tag) is available. This protein tag has been derived from mammalian DNA repair protein O^6-alkylguanin-DNA-alkyltransferase (AGT). AGT transfers the alkyl group of its substrate - O^6-benzylguanine (BG) into one of its cystein residues. A fluorescent derivates of BG can be also used as substrate. If a protein is fused with AGT it can be enzymatically labelled with fluorescent dye *in vivo* (Keppler et al., 2003; Keppler et al., 2004a; Keppler et al., 2004b). SV 40 virus labelled with BG was used for tracking in ER and cytosol. BG labelled SV40 was literally attached to the ER or cytosol expressed SNAP protein (Geiger et al., 2011). Also, they used this method for adenovirus one year later (Geiger et al., 2013). This method was also used for labelling HIV gag protein with SNAP-tag and then labelled it *in vivo* with BG derivatives. A decade later method similar to SNAP-tag was engineered later - the CLIP-tag. Difference in these methods is used substrate. CLIP-tag uses as substrate O^2-benzylcytosine and its derivatives (Gautier et al., 2008).

Fluorescent protein tag can be designed to be located inside the virus. This design avoids the surface modification of virus and thus provides natural way of virus entry. Tag fused with proteins oriented inside the viral particle can be problematic. The first is that capsid will not be able to assemble if we use to large tag. The second is with quenching of fluorescent tags if they are too close. And last, if we "encapsidate" fluorescent protein it may result in particles without genome inside. This could be caused by lack of space inside or localization signal for DNA on viral proteins is not accessible (Gilbert et al., 2004). In HBV was successfully created linker between fluorescent proteins to avoid quenching of fluorophores. The fluorescent protein tags were on the surface of capsid but HBV is enveloped virus so it is on the edge between "encapsidated" and surface tag (Yoo et al., 2012).

2.3.2 Quantum dots (QDs)

Another way of labelling virus is utilization of Quantum Dots (QDs). QDs are small nanoparticles made of semiconductors with fluorescent properties. The colour of QDs is determined by its size (5-50 nm). QDs are also electrodense material so they can be used in electron microscopy or correlative microscopy. Their utilization *in vivo* is problematic due to its solubility in aqueous solution required by capsid proteins and pH of the solution. For the life cell imaging can be toxic to its surrounding because of release of reactive species during exposure to light (Derfus et al., 2004; Dixit et al., 2006).

Genome is encapsidated in viral capsid naturally. This phenomenon is used to encapsidate therapeutic cargo (e.g. gene therapy, drugs). Another utilization of this

phenomenon is packaging fluorophores - the quantum dots (QD). Encapsidation of QDs in the particle means partially or completely replacing viral genome with QDs. Regardless of abolished infectivity of these particles, they can still be used for tracking of viral entry in the cell. Li and his colleagues (2009) successfully encapsidated QD in SV40 virus like particle (VLP) which they used to study SV40 trafficking towards ER and its accumulation in ER. Advantage of SV40 self-assembly was used in this study. Later, it has been shown that QDs help the self-assembly of SV40 even in dissociation buffer where the capsids should be disassembled although the capsids have smaller diameter (25 nm) (Gao et al., 2013). QDs were specifically encapsidated in plant virus called Red Clover Necrotic Mosaic Virus (RCNMV). RCNMV pack its two molecules of ssRNA genome via structural specific interaction (Basnayake et al., 2009). Complementary ssDNA to the genomic ssRNA was attached to the QD. Hybridized ssDNA with ssRNA generates secondary structure recognized by capsid proteins and QDs were specifically encapsidated (Fig. 2.4) (Loo et al., 2007). A similar strategy of specific encapsidation was applied to enveloped virus. QDs were encapsidated into vesicular stomatitis virus glycoprotein pseudotyped HIV-1 based lentivirus *in vivo*. Hybridized (*in vitro*) genomic RNA with DNA conjugated with QD is co-transfected into cell with packaging plasmids which are used for virus production (Zhang et al., 2013b).

QDs can be encapsidated not only in VLPs but also in infectious virus. There are three types of conjugation of QDs with virus which can preserve viral capacity to produce progeny. In this case are viruses labelled with QDs via conjugation chemistry, bio-affinity and in few cases via electrostatic interaction. Initial work where was QDs bioconjugated used cross-linkers on QDs and on non-enveloped Adeno-associated virus. QDs were linked to the virus via carbodiimide couple reaction which involves carboxylic moieties on QD and primary amines from lysine residues on the viral capsid (Joo et al., 2011). Conjugation of QDs can be achieved via click chemistry with dibenzocyclooctynes modified QDs and azide modified virus (Hao et al., 2012). QDs labelled virus is really useful tool. An influenza virus has been labelled with QDs via bioorthogonal click chemistry and used *in vivo* for imaging. This time *in vivo* means in a mouse (Fig. 2.5) (Pan et al., 2014).

Bio-affinity conjugation is based on biotinilated virus and streptavidin modified QDs. A short (15 amino acids long) acceptor peptide (AP) is often used for biotinilation of virus. AP is usually expressed on cellular membrane. Enveloped viruses like HIV can acquire APs from cellular membrane when it buds from cell. APs on viral envelope are then biotinylated *in vitro* and conjugated with QDs modified with streptavidin (Joo et al., 2008). A “shorter” version of this method has been published. Biotin is directly attached on cell surface and cells are infected with virus. Isolated virus is coated with biotin modified envelope which is *in vitro*

conjugated with streptavidin modified QDs (Fig. 2.4)(Huang et al., 2012; Zheng et al., 2014). Some enveloped viruses can be directly biotinilated *in vitro* (Liu et al., 2011; Liu et al., 2012b). A method has been developed in baculovirus (enveloped insect virus) to label nucleocapsid. Virus was produced from recombinant bacmid in which gene for nucleocapsid protein has been fused with AP and biotinilated *in vivo*. Virus was isolated and labelled *in vitro* (Wen et al., 2014). A native VSV (vesicular stomatitis virus) with surface sialic acid conjugated with phenylboronic acid modified QDs provide simple, reversible labelling. This method is biocompatible however it cannot be used to label *in vivo* because cell contains its own sialic acid (Huang et al., 2014)

Work in which authors conjugate QDs electrostatically are no so common. QDs are modified with positively charged molecule which is conjugated on cellular membrane with negatively charged molecules (Chen et al., 2010; You et al., 2006).

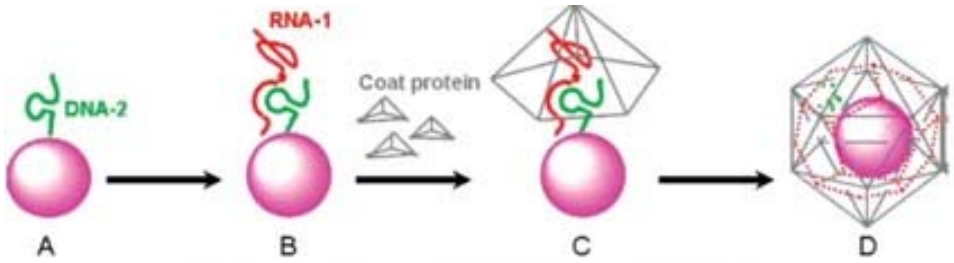


Figure 2.4: Encapsidation of QD in VLPs. A: Specific encapsidation of QDs in non-enveloped virus via DNA and RNA hybridization. B: Specific encapsidation of QDs in enveloped virus based on RNA genome and DNA probe hybridization (Loo et al., 2007).

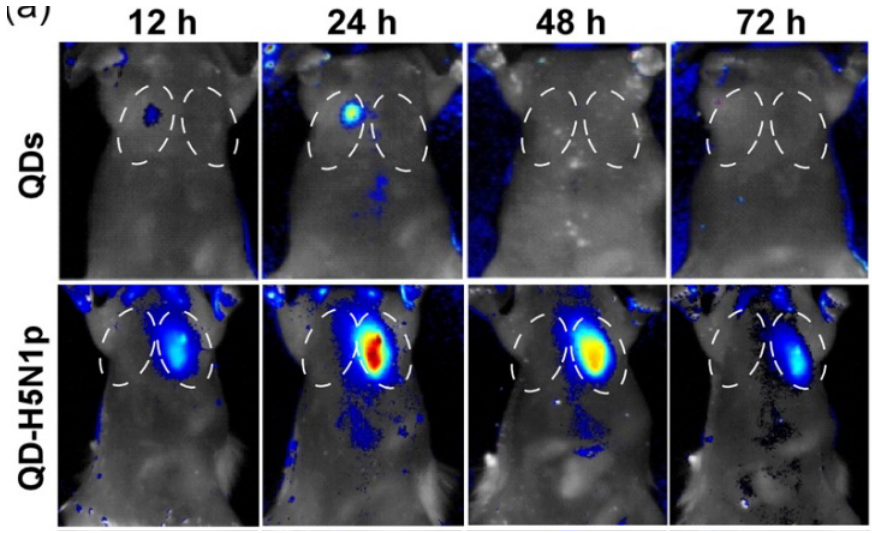


Figure 2.5: Imaging QDs labelled influenza virus inhaled by mouse. In first row is inhaled QDs alone. In second row is inhaled influenza virus labelled with QDs. Adapted from Pan et al. (2014).

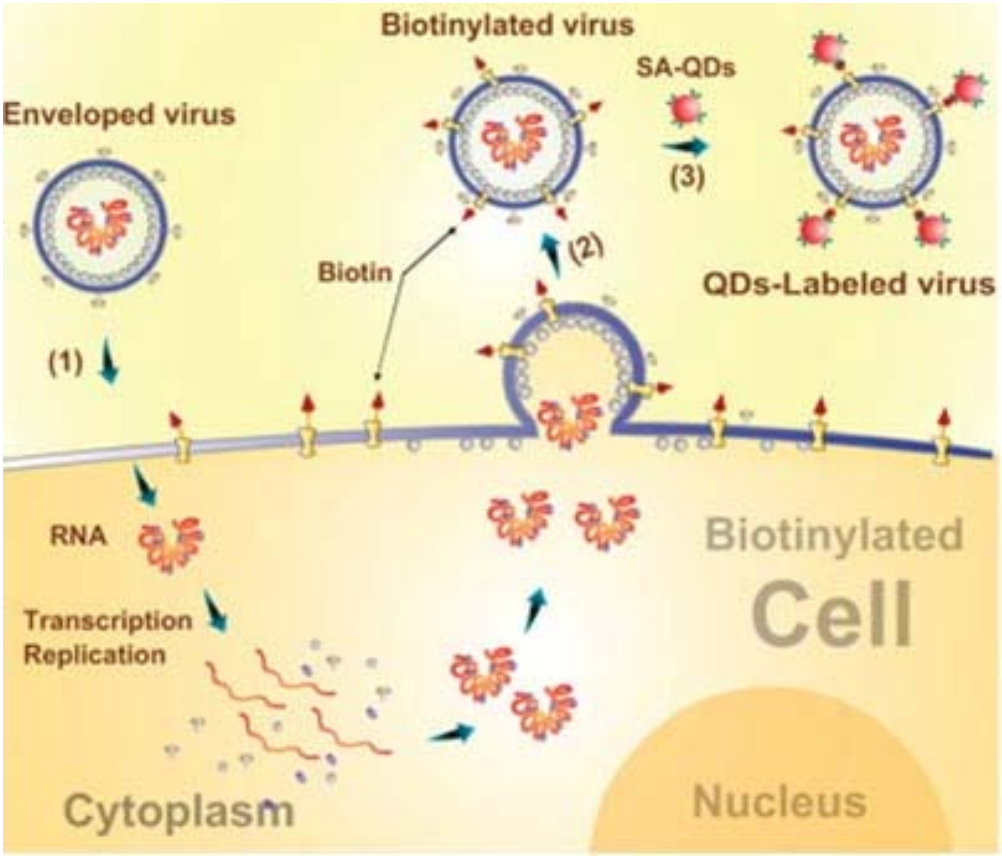


Figure 2.6: Illustrations of different conjugation methods of virus and QDs. A: Biotinilation of envelope of enveloped virus. Cells are infected with enveloped virus. After infection, cells are biotinilated and virus is budding from cells with biotin. The virus is then labelled with streptavidin modified QDs *in vitro*. Adapted from Zheng et al. (2014)

2.3.3 Fluorescent dyes

QDs are not the only fluorophores which can be conjugated with virus. Also fluorescent chemical compounds such as Alexa Fluor dyes, FITC, rhodamine can be conjugated. QDs are very useful because of their photostability but on the other hand, they are highly cytotoxic (Derfus et al., 2004). One of the first experiments that study virus-host interaction with real-time life-cell imaging was done in 1990 by Georgi and his colleagues. They successfully labelled reovirus with rhodamin B or FITC. Another group has successfully labelled adenovirus (Defer et al., 1990; Leopold et al., 1998; Persson et al., 1983). A complex dynamic study of adenoviral vector and adeno-associated virus labelled with fluorescent dyes was achieved (Leopold et al., 1998; Seisenberger et al., 2001). Since then, many papers have been published and even a kit for labelling exists. A development of bioorthogonal azide-alkyne cycloaddition offers a new way to label viruses *in vivo*. This method is highly specific and creating no background. A pioneer study in this field used Cowpea mosaic virus only as

macromolecule to enhance fluorescence of azide labelled cellular surface glycans (Washington-Hughes et al., 2013). These viruses have not been used for live cell imaging yet nor for trafficking research but the message is clear. We can label viruses or virus like particles with high specify without affecting structure and thus offers a new powerful tool for exploring virus-host interaction or immunological responses.

Fluorescent dyes can be used as cargo in VLPs. Although these particles are not infectious, they can still provide information about trafficking. The approach to encapsidate dye is similar to encapsidation of QDs. Fluorescent molecule is tagged to the polymer which is encapsidated. VLP can increase the size with encapsidated fluorescent labelled polystyrene bead (Cadena-Nava et al., 2011). Another way to encapsidate dye in particles is use of disassembly and reassembly of particles with dye (Loo et al., 2008). A directed encapsidation of fluorescent proteins can be achieved with non-covalent binding to the capsid via coiled-coil motif. First motif is attached to capsid protein and second motif is attached to the fluorescent protein. So called E-coil and K-coil are negatively charged with glutamic acid (E-coil) or positively charged with lysine (K-coil) (Minten et al., 2009; Rurup et al., 2014). The encapsidation of fluorescence dye (protein) is performed by *in vitro* assembly of capsids.

2.3.4 Hydrophilic dyes

In the structure of enveloped viruses, we can label lipid membrane with specific probe. A lipophilic fluorescent probe 1,1´-dioctadecyl-3,3,3´,3´-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) or its analogues DiI, DiO, DiR, DiA has been used to label dengue virus membrane, HCV membrane and influenza membrane. There is one major advantage of this approach - DiD is hydrophobic and it spontaneously integrates in lipid membrane. Individual virus entry, trafficking and membrane fusion can be tracked with this method (Ayala-Nunez et al., 2011; Coller et al., 2009; van der Schaar et al., 2007). The kinetics of membrane fusion and entry of Dengue virus was studied with DiD labelled membrane (van der Schaar et al., 2008). Another labelling of virus membrane has been published for influenza. Virus membrane labelled with rhodamine 110 octadecyl ester was used to study kinetics of the membrane fusion (Floyd et al., 2008).

2.3.5 Genome labelling

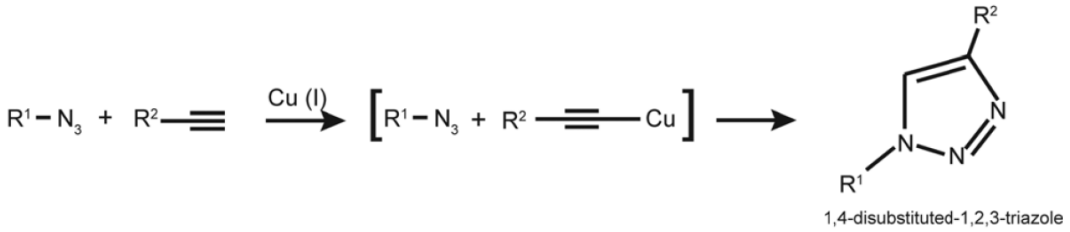
There is one common aspect for all viruses across viral families - the infectious part of virus is genome. Tracking of viral genome allows us to really see the whole infective pathway. Labelled genome would allow us to observe all interactions between virus and its host. First work where genome was labelled appeared a decade ago when Babcock and his colleagues (2004) used isolated influenza RNA genome in form of ribonucleoproteins which were labelled with amine reactive dye and inserted in cells with microinjection. However this study only observes transport through nuclear membrane and the genome did not get inside the cell in natural way. To this date only RNA viruses have been tracked. Liu and his collegues tested 20 RNA intercalating dyes and only one - Syto 82 provided desired characteristics (photostability, preserving the infectivity and without cytotoxity) for *in vivo* tracking (Liu et al., 2012a). A new intercalating dye based on Luminescent Ru(II) complexes $[Ru(phen)_2(dppz)]^{2+}$ has been developed for *in vivo* tracking. Cell infected with vaccinia virus were treated with these complexes. Vaccinia virus replicates its DNA cytoplasm and thus these complexes are intercalated into the DNA (Huang et al., 2013). This dye was shown to intercalate in dsDNA of baculovirus which is replicated in the nucleus (Zhou et al., 2012). Labelling genome is problematic. When viral DNA or RNA is labelled it's simultaneously labelled cellular nucleic acids. This could result to cell death before the viral replication cycle is complete.

A labelled non-viral DNA can be encapsidated into VLPs. This method is useful for tracking DNA delivery utilized by VLPs for example therapeutic DNA delivery in cancer cells. Produced virus (cucumber mosaic virus) is *in vitro* dissembled. Proteins and DNA are separated and DNA labelled with Fluorescent dye (streptavidin-biotin conjugation) is encapsidated into particles (Lu et al., 2012).

Viral DNA can be labelled by incorporation of thymidine analogues in it. To label MPyV genome we use two thymidine analogues. First is 5-Ethynyl-2´-deoxyuridine (EdU) and second is 5-bromo-2´-deoxyuridine (BrdU). One of the aims of this thesis is establishing detection for EdU of BrdU virus in fixed samples. BrdU is detected with an antibody and EdU is detected by click chemistry. Click chemistry is term for joining small units with heteroatom link (C-X-C) (Kolb et al., 2001). In biology is mostly used azide-alkyne Huisgen cycloaddition. EdU is analogue with alkyne group which is clicked to the Alexa Fluor with azide group (Cavanagh et al., 2011). The reaction we use is catalyzed with copper which is toxic so we detect EdU only on fixed samples (reaction scheme on Fig. 2.7) but a click chemistry which can be used *in vivo* has been developed. So-called bioorthogonal (inert components to biological surrounding) cycloaddition for click reaction is reviewed in Jewett and Bertozzi (2010). The advantage of this

labelling is that we label genome and thus we can track the infectious part of virus. Second advantage is that EdU and BrdU are incorporated naturally by cellular polymerases. The reason why we use two analogues is that BrdU can be labelled by antibody in sections for electron microscopy and EdU detection for light microscopy is faster, easier and more specific.

A



B

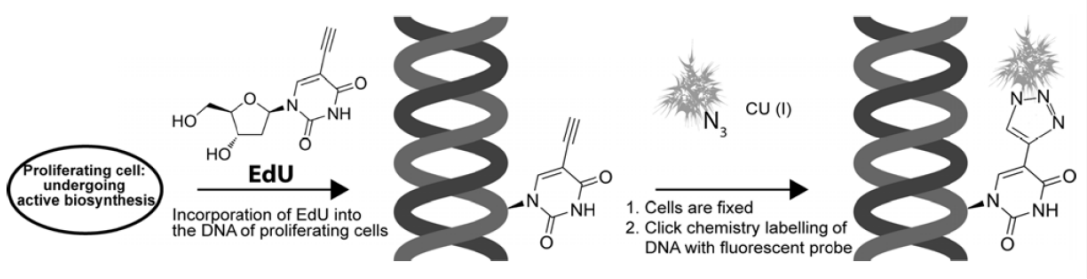


Figure 2.7: A: Scheme of copper [Cu(I)]-catalyzed reaction. **B**: EdU is incorporated into DNA during S-phase. Cells are fixed and EdU is covalently bound with copper [Cu(I)]-catalyzed cycloaddition reaction to fluorescent probe. Adapted from Cavanagh (2011).

2.3.6 Labelling with multiple systems

Multiple viral compounds can be labelled with multiple methods. For example, in baculovirus dual-labelling was performed. Lipid envelope was conjugated with QDs and nucleocapsid was fused with GFP. Labelling of envelope and nucleocapsid allows tracking dissociation between envelope and nucleocapsid as well as interaction with cell (Zhang et al., 2013a). Streptavidin-biotin conjugated QDs were compared to DiD labelling of influenza virus envelope. Labelling efficiency of DiD is very low and when immunolabelling was performed DiD was easily washed in process. Furthermore in this study was used simultaneous labelling of envelope (QDs and DiD) and RNA genome (Syto 82 intercalating dye) to compare transport of the envelope and genome (Liu et al., 2012a). Another study performed in baculovirus used genetically fused GFP with enveloped protein and DNA labelled with metal complexes (see chapter 3.2.1.4) to distinguish traffic of viral envelope and viral genome (Zhou et al., 2012). Infectious vaccinia virus had successfully labelled genome by metal complexes and envelope containing azides (which is clicked *in vitro*). The elegance of this kind of dual labelling is in the

way the virus is produced. Dual-labelling is performed during natural life cycle of virus without any genetic modification or any *in vitro* modification with fluorophores (Fig. 2.8) (Huang et al., 2013).

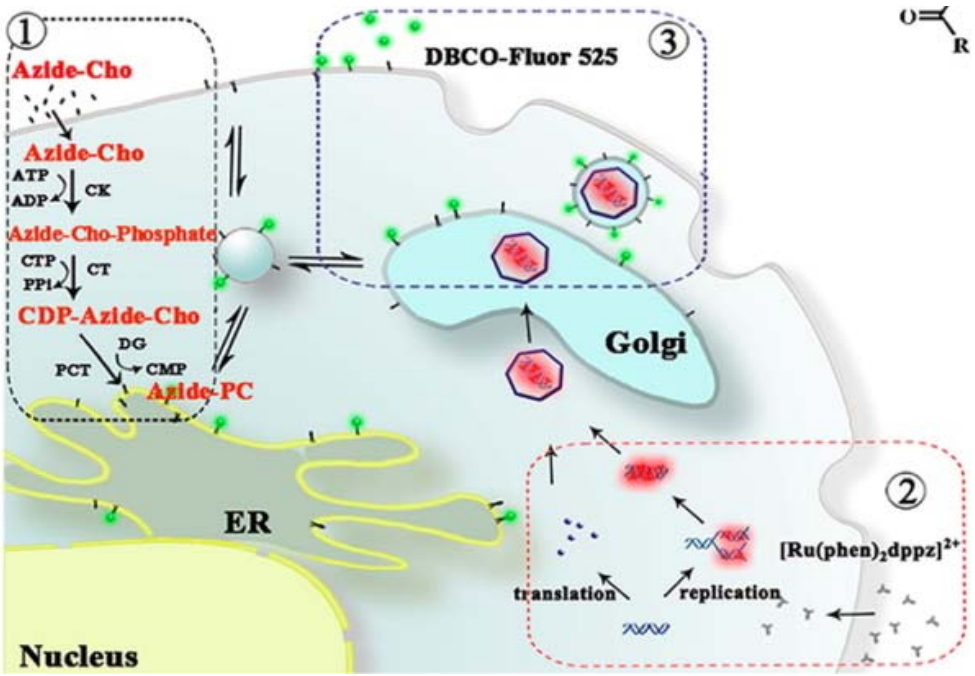


Figure 2.8: Illustration of dual labelling of enveloped virus. DNA was labelled with intercalating dye containing metal complexes. On the cellular membrane were incorporated choline analogues with terminal azide (Azide-Cho). Azide-Cho was labelled with florescent molecule with strain-promoted azide–alkyne cycloaddition which is bioorthogonal. Adapted from Huang et al. (2013).

3. Aims

Early phase of polyomavirus infection proceeds in the several steps that determine the outcome of viral infection. Some of them, such as virion binding to its receptor and transport of virions from membrane towards the nucleus, have been recently intensively studied for several polyomaviruses. The others, like delivery of the genome into the cell nucleus, remain enigmatic.

Minor proteins seem to play a vital role in most of these processes. Viral mutants lacking either VP2 or VP3 are non-infectious and pseudovirions composed of VP1 alone deliver heterologous DNA for gene expression very inefficiently.

Therefore, the long term goal of our research is to determine the role of minor proteins in the early steps of infection, especially during the delivery of viral genome into the nucleus. We study minor proteins involvement in critical events of viral infection with series of mutants with lesions in minor proteins. Some of these events can be discerned by biochemical means, but our research mostly relies on fluorescent and electron microscopy techniques. While the virion tracking from membrane towards endo-lysosomal compartments or endoplasmic reticulum can be detected with antibodies against viral capsid proteins (or virions with labelled capsid). The visualization of viral genome translocation into the nucleus requires genome labelling and/or advanced techniques in electron microscopy.

With three distinct aims this thesis fits in the long term goal of our research. The specific aims are:

I. **To produce and characterize the viral mutant (designated as VP1_only) lacking both minor proteins. For efficient mutant production, the novel system based on Cre-LoxP recombination should be used and its utility for production of non-viable mutants (e. g. mutants with lesions in minor proteins) verified.**

II. **To produce and characterize polyomavirus with genome modified by thymidine analogues (BrdU or EdU) and subsequently establish a method of genome labeling and detection during early steps of infection.**

III. **To verify the previous results from our laboratory that high pressure freezing fixation of samples for electron microscopy can be used for visualization of virion translocation into the nucleus.**

4. Material and methods

4.1 Materials

4.1.1 Cell lines

3T6 - adherent cell line of Mouse Embryonic Fibroblasts, (ATCC - CCL-96)
WOP - adherent cell line of Mouse Embryonic Fibroblasts (3T3 cell line), stably transformed with Mouse Polyomavirus (producing only LT antigen) (P. Amati, Universita La Sapienza, Rome)
NIH 3T3 - adherent cell line of Mouse Embryonic Fibroblasts (ATCC - CRL-1658)
T2 - adherent cell line of Mouse Embryonic Fibroblast derived from 3T6, stably transformed with Cre recombinase (pNit-Cre) (Hron et al., 2013)

4.1.2 Bacterial strains

XL1 Blue - *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lac*IqZΔ*M15* Tn*10* (Tetr)] (Stratagene)

4.1.3 Virus

Mouse Polyomavirus BG Strain (B. Griffin, Royal Postgraduate School, London)

4.1.4 Vectors

pMJG - Recombinant plasmid derived from pBR322 with Mouse Polyomavirus genome (BG strain) resistant to ampicilin (Krauzewicz et al., 1990).
pMJ-VP1 - Recombinant plasmid derived from pMJG which is not expressing VP2 and VP3 (BG strain). Plasmid is resistant to ampicilin (H. Španielová, Charles University in Prague, Prague).
pBS-PyI - Recombinant plasmid derived from pBluescript-SK with Mouse Polyomavirus genome (BG Strain) and two LoxP sites in intron region of T antigens resistant to ampicilin (Hron et al., 2013).

pPuro.Cre empty - Recombinant plasmid for production of Cre recombinase in mammalian cells resistant to ampicilin (Kumar et al., 2008).

pCALNL-DsRed - expression vector for fluorescent protein DsRed induced by Cre recombinase resistant to ampicilin (Matsuda and Cepko, 2007).

pGL3 control - Control plasmid used as mock DNA for transfection (Promega).

RV1 - pGL3 control plasmid with inserted regulatory region of MPyV (Spanielova et al., 2014).

4.1.5 Antibodies

4.1.5.1 Primary antibodies

αPyVP1-D4	Mouse monoclonal IgG against Mouse Polyomavirus VP1, dilution 1:10 - 1:50 (prepared by Alena Morávková)
αPyVP1-A	Mouse monoclonal IgG against Mouse Polyomavirus VP1, dilution 1:100 (Forstova et al., 1993)
αPyVP2/3 2C8	Mouse monoclonal IgG against Mouse Polyomavirus VP2 and VP3 common region, not diluted (prepared by Alena Morávková)
αPyLT1	Rat monoclonal IgG against Mouse Polyomavirus LT, dilution 1:100 (Dilworth and Griffin, 1982)
αPyC1	Rat monoclonal IgG against Mouse Polyomavirus T antigens common region, dilution 1:2 (Dilworth and Griffin, 1982)
αPyC4	Rat monoclonal IgG against Mouse Polyomavirus T antigens common region, dilution 1:2 (Dilworth and Griffin, 1982)
αPyACIID7	Mouse monoclonal IgG against Mouse Polyomavirus T antigens common region, dilution 1:50 (Dilworth and Griffin, 1982)
αBrdU	Rat monoclonal IgG against BrdU (Abcam ab6326)
αPyVP1 - rabbit	Rabbit polyclonal IgG against Mouse Polyomavirus VP1 protein, dilution 1:10 or 1:20
αcavelin-1	Rabbit polyclonal IgG against Caveolin-1, dilution 1:20 (Santa Cruz Biotechnology)
anti rab 5	Rabbit monoclonal IgG against Rab5, dilution 1:20 (Abcam ab109534)
antirab 7	Rabbit monoclonal IgG against Rab7, dilution 1:20 (Abcam ab137029)
anti rab 11	Rabbit IgG against Rab11, dilution 1:20 (Invitrogen)
anti NPC	Mouse monoclonal IgG to Nuclear Pore Complex Proteins (Abcam Ab 24609)

4.1.5.2 Secondary antibodies

αmouse Alexa Fluor 488	Donkey polyclonal IgG against mouse Ig conjugated with Alexa Fluor 488, dilution 1:1000 (Molecular Probes)
αmouse Cy3	Goat polyclonal IgG against mouse Ig conjugated with cyanine dye (Cy3), dilution 1:1000 (Invitrogen)
αmouse HRP	Goat polyclonal IgG against mouse Ig conjugated with horse radish peroxidase, dilution 1:1000 (BioRad)
αrat Alexa Fluor 488	Donkey polyclonal IgG against mouse Ig conjugated with Alexa Fluor 488, dilution 1:1000 (Molecular Probes)
αrat Alexa Fluor 546	Goat polyclonal IgG against mouse Ig conjugated with Alexa Fluor 549, dilution 1:1000 (Molecular Probes)
αrat Alexa Fluor 594	Donkey polyclonal IgG against mouse Ig conjugated with Alexa Fluor 594, dilution 1:1000 (Molecular Probes)
αrat HRP	Goat polyclonal IgG against mouse Ig conjugated with horse radish peroxidase, dilution 1:300 (Sigma-Aldrich)
αmouse immunogold conjugate 10 nm	Immunogold conjugate Goat anti rabbit IgG:10 nm (BBI International
αmouse immunogold conjugate 5 nm	Immunogold conjugate Goat anti rabbit IgG:10 nm (BBI International
αmouse immunogold conjugate 10 nm	Immunogold conjugate Goat anti rabbit IgG:10 nm (BBI International
αrabbit immunogold conjugate 5 nm	Immunogold conjugate Goat anti rabbit IgG:10 nm (BBI International

4.1.6 Stains

DAPI (4,6-diaminido-2-phenylindol) - diluted 1:500 in 50% glycerol

GelRed Nucleic Acid Stain (Biotium) - dilution 1:10000

Ethidium bromide (Calbiochem) - 0.5 ng/ml

Alexa Fluor 594-Azide (Molecular Probes)

Alexa Fluor 488-Azide (Molecular Probes)

4.1.7 Enzymes

4.1.7.1 DNA polymerases

Pwo DNA polymerase (Roche)

Deep Vent (exo$^-$) DNA polymerase (Thermo Scientific)

Vent DNA polymerase (Thermo Scientific)

DreamTaq DNA polymerase (Thermo Scientific)

4.1.7.2 DNA ligases

T4 DNA ligase (Fermentas)

4.1.7.3 Restriction endonucleases

EcoRI (Fermentas)

EcoRV (Fermentas)

NarI (New England Biolabs)

SalI (Fermentas)

XhoI (Fermentas)

4.1.8 Markers

4.1.8.1 DNA Marker

O´GeneRuler 1 kb Plus DNA Ladder, ready-to-use (Thermo Scientific) applied in all DNA electrophoresis.

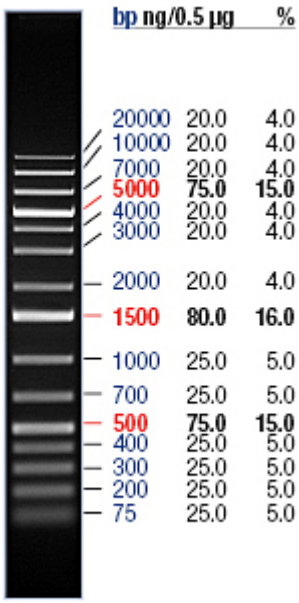


Figure 4.1: O´GeneRuler 1 kb Plus DNA Ladder, ready-to-use (Thermo Scientific) fragments with base-pair indication of bands. Adapted from: http://eshop.biogen.cz/ogeneruler-1-kb-plus-dna-ladder-ready-to-use

4.1.8.2 Protein Marker

Spectra™ Multicolor Broad Range Protein Ladder (Thermo Scientific). Applied in all protein electrophoresis (SDS-PAGE)

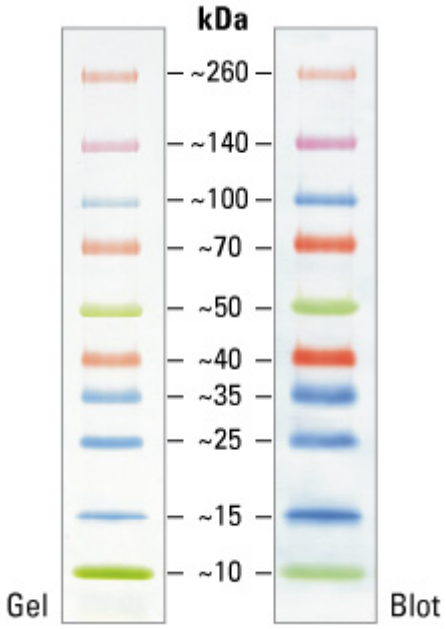


Figure 4.2: Spectra™ Multicolor Broad Range Protein Ladder (Thermo Scientific) with kDa indication of bands. Adapted from: https://www.lifetechnologies.com/order/catalog/product/26634

4.1.9 Primers

MPyV-OriDetection-Fw	5´-CCA GGC CTA GAA TGT TTC CA - 3´	Primer for amplification of MPyV Ori used in qPCR. It binds to position 5237 - 5256 in MPyV genome
MPyV-OriDetection-Rw	5´-GAT GGT GGT GAG GCT GAA AT - 3´	Primer for amplification of MPyV Ori used in qPCR. It binds to position 174 - 155 in MPyV genome
H20-1PyV-5121Rw	5´-TGC GGT CAG TTA GTC ACT TC - 3´	Primer used for sequencing inserted mutated VP2 and VP3 genes. Used for amplification of Ori sequence from plasmid RV1. It binds to position 5121 - 5101 in MPyV genome.
RVprimer_pGL3	5´ -CTA GCA AAA TAG GCT GTC CC- 3´	Primer used for amplification of Ori sequence from plasmid RV1.

4.1.10 Frequently used solutions

Phosphate buffer saline (PBS)	1.37 M NaCl; 0.027 M KCl; 0.100 M $Na_2HPO^4 \cdot 12\ H_2O$; 0.018 M KH_2PO_4; pH = 7.4
Buffer B	0.15 M NaCl; 0.01 M Tris-HCl pH = 7.4; 0.01 mM $CaCl_2$
Blocking Solution for IF	1) 0.25% (w/V) gelatine from porcine skin; 0.25% (w/V) BSA in PBS 2) 0.2% (w/V) teleostean gelatine in PBS
Fixing Solution for IF	3,7 % Formaldehyde
Permeabilization Solution for IF	0,5 % (V/V) Triton X-100
TAE	40 mM Tris; 20mM acetic acid; 1mM EDTA pH =8
TBE (0.5x)	44 mM Tris; 44 mM boric acid; 1mM EDTA pH =8
Chemiluminescence reagent	Solution A) 100 mM Tris-HCl pH = 8.5; 0.36 mM p-coumaric acid; 2.5 mM Luminol Solution B) 100 mM Tris-HCl pH = 8.5; 0.00045% H_2O_2 Solution A and B mixed 1:1 just before use
TE buffer	10 mM Tris-HCl pH = 8; 1 mM EDTA pH = 8

4.1.11 Culture media

4.1.11.1 Bacterial culture media

LB Medium	1% bacteriological Peptone(w/V); 0.5% Yeast extract (w/V); 1% NaCl (w/V)
Nutrient Broth NO.2	1%`Lab-Lemco' powder; 1% bacteriological peptone; 0.5% NaCl
SOC Medium	2% bacteriological Peptone(w/V); 0.5% Yeast extract (w/V); 10 mM NaCl; 2.5 mM KCl; 20 mM glucose; 10 mM $MgCl_2$; 10mM $MgSO_4$
TPN Medium	2% bacteriological Peptone(w/V); 0.5% Yeast extract (w/V); 0.5% NaCl (w/V)

4.1.11.2 Cell culture media

DMEM serum supplemented	DMEM (Dublecco´s Modified Eagle´s Medium, Sigma-Aldrich); 10% FBS (Fetal Bovine Serum)(Gibco); 4mM L-Glutamine (Gibco);
DMEM serum supplemented with antibiotics	DMEM (Dublecco´s Modified Eagle´s Medium, Sigma-Aldrich); 10% FBS (Fetal Bovine Serum)(Gibco); 4mM L-Glutamine (Gibco); Antibiotics mixture (100 U/ml of penicillin; 0.02 mg/ml of streptomycin; 0.05 mg/ml of amphotericin B
DMEM serum-free	DMEM (Dublecco´s Modified Eagle´s Medium, Sigma-Aldrich); 4mM L-Glutamine (Gibco)
1640 RPMI	1640 RPMI (Sigma-Aldrich); 5% FBS

4.1.12 Chemicals

β-Mercaptoethanol (Serva)
35% Hydrochloric Acid (Lach:ner)
5-Bromo-2'-deoxyuridine (Sigma-Aldrich)
5-Ethynyl-2'-deoxyuridine (Molecular Probes)
5-Ethynyl-2'-deoxyuridine 5'-triphosphate (Jena Bioscience)
5-Bromo-2'-deoxyuridine 5'-triphosphate (Sigma-Aldrich)
70% Perchloric Acid
96% Ethanol (Lach:ner)
Acetic Acid (Penta)
Acetone (Lach:ner)
Acrylamide (Serva)
Agar 100 Resin Kit (Agar Scientific)
Albumin from Bovine Serum (Sigma-Aldrich)
Amonium Persulfate (Serva)
Ampicillin (BIOTIKA)
Aprotinin (Sigma-Aldrich)
Bayol F Paraffin Oil (Serva)
Bisacrylamide (Serva)
Boric Acid (Serva)
Bromphenol Blue (Výzkumný ústav přírodních léčiv, Praha 9)
Calcium Chloride (Sigma-Aldrich)
Caesium Chloride (Serva)
Chloroform (Lachema)
Coomassie Brilliant Blue G250 (CBBG) (Serva)
Deoxyribonucleoside Triphosphates – dATP, dTTP, dCTP, dGTP (Fermentas)
Dimethylsulfoxid – DMSO (Sigma-Aldrich)
Dithiothreitol - DDT (Roche)
Ethylendiamintetraacetate Disodium – EDTA (Serva)
Gelatin from porcine skin (Sigma-Aldrich)
Gelatin from cold water fish skin (Teleostean Gelatin)
Glycerol (Penta)
Glycine (Serva)
GlutaMax (L-alanyl-Lglutamine) (Gibco)
Grutaraldehyde (Sigma-Aldrich)
Hydrogen Peroxide (Sigma-Aldrich)
Isoamyl alcohol (Lach:ner)
Isopropyl alcohol (Lach:ner)
Luminol (Sigma)
Lowicryl HM20 resin (Electron Microscopy Sciences)
LR White (London Resin Company Ltd.)
Magnesium Chloride (Lachema)
Magnesium Sulfate (Lach:ner)

Methanol (Lach:ner)
N,N,N´,N´ - Tetramethylethylendiamin – TEMED (Sigma-Aldrich)
Nutrient Agar N°2 (Biolife)
Osmium tetroxide (Electron Microscopy Sciences)
p-Coumaric Acid (Sigma-Aldrich)
Paraformaldehyde – PFA (Sigma-Aldrich)
Peptone Bacteriological (Biolife)
Phenol (Sigma-Aldrich)
Potassium Chloride (Lachema)
Propylene Oxide (Sigma-Aldrich)
Protease Inhibitor Cocktail Tablets EDTA-free (Roche)
SeaPlaque Agarose (FMC Bioproducts)
Sodium Acetate (Sigma-Aldrich)
Sodium Cacodylate (Agar Scientific)
Sodium Chloride (Lach:ner)
Sodium Dodecyl Sulphate – SDS (Sigma-Aldrich)
Sodium Hydroxide (Penta)
Sucrose (Lach:ner)
Tris(hydroxymethyl)aminomethane TRIS (Serva)
Triton X-100 (Serva)
Uranyl Acetate (Electron Microscopy Sciences)
Yeast Extract (IMUNA)

4.2 Machines and equipment

3130 Genetic Analyzer Sequenator
3K30 Centrifuge, 12171, 12154-H, 19776-H rotors (Sigma)
ABBE Refractometer (Carl Zeiss Jena)
Amaxa Nucleofector (Amaxa Biosystems)
Agarose electrophoresis horizontal apparatus multiSub Mini (Cleaver)
CO2 thermostat (Forma Scientific)
Confocal Microscope Leica TCS SP-2
Confocal Microscope Leica TCS SP-5
Duomax 1030 Shaker (Heidolph)
Eppendorf centrifuge 5810R (Eppendorf)
Gene Pulser Apparatus Electroporator (Bio-Rad)
Helios β Spectrophotometer (Thermo Electron)
Hybridization Incubator Model 100 (Robbins Scientific)
Inverted Light Microscope (Carl Zeiss Jena)
JEOL JEM-1011 Electron Microscope with Valeta CCD camera
Laminar Biohazard Cabinet (Forma Scientific)
Light Cycler 480 II (Roche)
Mastercycler EPgradient S PCR Cycler (Eppendorf)

Microfuge Lite 16 Microcentrifuge, A46544 rotor (Beckman)
Microfuge R Microcentrifuge, F241.5 rotor (Beckman)
Mini-PROTEAN Tetra Cell apparatus for SDS-PAGE and Western blotting (Bio-Rad)
MiniSpin plus Microcentrifuge, IL 016 rotor (Eppendorf)
MPW 300 centrifuge (MPW Med. Instruments)
ND – 1000 Spectrophotometer (NanoDrop) 39
Olympus BX-60 Fluorescence Microscope (Olympus)
Olympus CK40 Inverted Light Microscope (Olympus)
Olympus IX 71 Inverted Fluorescence Microscope (Olympus)
Optima TM L-90K Ultracentrifuge - SW 28, SW 41 and SW 55Ti rotors (Beckman)
Orbi-Safe TS Shaking Incubator (Gallenkamp)
Orbital Shaker Shaking Incubator (Forma Scientific)
PAGE vertical apparatus Hoefer Mighty Small II (Hoefer)
PCR Cabinet (ESCO)
Qubit Fluorometr (Invitrogen)
Shaker 30 (Labnet)
Soniprep 150 Sonicator (Schoeller)
Stratalinker UV Crosslinker (Gemini B.V.)
SUB Water Bath (Grant)
TCH 100 Thermostat (laboratorní přístroje Praha)
UP50H Sonicator (dr. Hielscher)
UV Transluminator (BioLum)
Varioskan Flash fluorometer (Thermo Scientific)
Vortex-Genie 2 (Scientific Industries)
Zeiss Observer A.1 Inverted Fluorescence Microscope (Zeiss)

4.3 Methods

4.3.1 Sterilization

All plastic equipment used for cell and bacterial culture was sterilized by manufacturer. Tips for automatic pipettes, microtubes and solutions were autoclaved (30 minute, 120 kPa, 127°). Non-autoclavable solutions were filtered with 220 nm filter. Glassware was sterilized by high temperature (160°C, 3 hours). Tweezers, cell spreaders and inoculation loops were sterilized by flaming.

4.3.2 Work with DNA

4.3.2.1 DNA Isolation

DNA samples were isolated with several methods depending on the sample.

4.3.2.1.1 Phenol-Chloroform extraction

DNA sample was diluted to the total volume of 400 µl before extraction to minimize losses of DNA. 400 µl (one volume) of phenol-chloroform-isoamylalcohol solution in ratio 25:24:1 was added to the sample and thoroughly vortexed. Sample was centrifuged for 5 minutes at 20,000 g. Upper aqueous phase was transferred to the fresh centrifuge tube. Another 400 µl (one volume) chloroform-isoamylalcohol (24:1) was added to the sample and thoroughly vortexed followed by centrifugation for 5 minutes at 20,000 g. Upper aqueous phase was transferred to the fresh centrifuge tube. Phenol-chloroform extraction is followed with ethanol precipitation.

4.3.2.1.2 Ethanol precipitation

Purified DNA sample was precipitated with ethanol to concentrate the sample. Two and half volumes of 96% ethanol and one tenth volume of 3 M sodium acetate (pH = 5) was mixed with the sample. DNA was precipitated at -20°C overnight or for 1 hour at -80°C. Precipitated DNA was centrifuged at 4°C for 30 minutes at 20,000 g. Ethanol was removed

without disturbing the DNA pellet which was washed with 70% ethanol. DNA was centrifuged at 4°C for 30 minutes at 20,000 g and ethanol was removed. Centrifugation was repeated for 3 minutes to remove as much ethanol as possible. DNA was dried at room temperature and dissolved in ddH_2O.

Viral genomes prepared *in vitro* by digestion and ligation in large volume (14 ml) were precipitated with two and half volume of 96% ethanol and one tenth volume of 3 M acetate (pH = 5). DNA was precipitated at -20°C overnight and centrifuged at 4°C for 4 hours at 3200 g in swinging rotor. DNA was washed with 1.5 ml of 70% ethanol and transferred to microtubes. The rest of the protocol is same as above.

4.3.2.1.3 DNA from purified virus

Viral inoculum in B-buffer with 10 mM $MgCl_2$ was treated with 20 U of DNase I and incubated for 1 hour at 37°C. After treatment, EDTA was added to final concentration 20 mM and DNase I was inactivated by incubating for 10 minutes at 80°C. Then Tris buffer (pH = 8) was added to the sample to final concentration 10 mM and it was treated with Proteinase K (final concentration 50 µg/ml) for 1 hour at 60°C to disassemble the viral capsids. After incubation, sample was treated with DTT (final concentration 25 mM) for 0.5 hour at 60°C to achieve complete degradation of viral capsids. The isolation of DNA continues with standard phenol-chloroform purification followed by ethanol precipitation.

4.3.2.1.4 Viral DNA from cell culture

Viral DNA from cells was isolated with Hirt method (Hirt, 1967). Cells were washed with PBS, trypsinized (0.25% trypsin in PBS) and resuspended in 2 ml of PBS. Cell were centrifuged for 10 minutes at 90 g. Supernatant was removed and 2.5 ml per dish (∅10 cm) of Hirt solution (0.6% SDS; 10 mM Tris-HCl pH = 7; 10 mM EDTA) with RNase A (20 µg/ml) was added. Cells were incubated in Hirt solution for 10 minutes and then, sodium chloride was added to final concentration 1 M. Cells were incubated overnight at 4°C. After incubation cells were centrifuged at 2,000 g for 45 minutes at 4°C. Supernatant was transferred to fresh microtubes and incubated with proteinase K (200 µg/ml) for 2 hours at 50°C. One volume of phenol was added to sample, thoroughly vortexed, and centrifuged at 8000 g for 10 minutes at 4°C. Upper phase was transferred to fresh microtubes and half the volume of TE buffer was added to the sample. The isolation continued with phenol-chloroform extraction and ethanol precipitation.

4.3.2.1.5 Cellular DNA from cell culture

DNA from cells was isolated with DNeasy Blood and Tissue Kit from Qiagen according to manufacturer´s instructions.

4.3.2.1.6 DNA from PCR reaction

DNA from PCR reaction was purified with NucleoSpin Gel and PCR Clean-up kit from Macherey - Nagel according to manufacturer´s instruction.

4.3.2.1.7 DNA from agarose gel

Separated DNA was cut out of gel and isolated with QIAEX II Gel Extraction Kit from Qiagen according to manufacturer´s instructions.

4.3.2.1.8 Plasmid isolation from bacterial culture

Plasmids were isolated from cultures by using endo-free commercial kits (Qiagen Endofree Plasmid Maxi Kit or Jet Star No Endo/Plasmid Purification Maxi Kit/20) according to manufacturer´s instructions.

4.3.2.1.9 Isolation of plasmid minipreparation

Bacterial culture was centrifuged for 1 minute at 20,000 g and pellet was resuspended in 250 µl of TE buffer. The suspension was cautiously mixed (by inverting the tube few times) with 250 µl of solution II (1% SDS, 0.2 M NaOH). Immediately after mixing with solution II, solution III (3 M Potassium acetate, 11.5% glacial acetic acid) was added and mixed with suspension by inverting the tube. Suspension was centrifuged for 5 minutes at 20,000 g. Supernatant was mixed by inverting the tube with 0.5 ml of 100% isopropylalcohol. Solution was centrifuged for 10 minutes at 20,000 g and pellet was washed with 200 µl 80% ethanol and centrifuged for 3 minutes at 20,000 g. Supernatant was removed and pellet was dried at room temperature. Dried pellet was resuspended in water (PCR grade) with RNase A (20 µg/ml).

4.3.2.2 Concentration determination

Concentration of DNA in sample was determined with Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) spectrophotometrically using absorbance at 260 nm wavelength or with Qubit fluorometer (Invitrogen) using Broad Range (BR) or High Sensitivity (HS) kits (both Invitrogen).

4.3.2.3 DNA electrophoresis

DNA was separated in 1% agarose gel. Agarose was dissolved completely by heating in appropriate volume of 0.5x TBE buffer or 1x TAE buffer. When the temperature decreased a nucleic acid stain was added to the agarose, properly mixed and poured into the electrophoresis chamber with comb. After solidification, the gel was transferred to the electrophoresis apparatus and overlaid with buffer (TBE or TAE depending on the gel). Samples mixed with loading dye (Bromphenol Blue or Orange DNA Loading Dye (Thermo Scientific)) were applied in the holes along with the marker. Electrophoresis ran at voltage 5V/cm. After separation of DNA, the gel was illuminated in UV transiluminator (BioLum) and a picture was taken.

4.3.2.4 Restriction Endonucleases Cleavage

Restriction mix for cleavage was prepared for each endonuclease specifically according to manufacturer´s instructions (Thermo Scientific; New England Biolabs). Volume of the mix was between 10 and 400 µl depending on purpose of cleaved DNA. The mix contained DNA, enzyme, specific buffer and ddH_2O. Reactions were incubated for 3 hours to overnight depending on enzyme and conditions in appropriate temperature.

Cleavage of mutated VP2 and VP3 from pMJ-VP1 and -type VP2 and VP3 from pBS-PyI was performed with NarI enzyme in NEBuffer to linearize the plasmid. DNA was purified with phenol-chloroform extraction and ethanol precipitation after digestion with NarI enzyme. Purified linearized plasmid was digested with EcoRV enzyme in 1x Tango buffer to excise mutated VP2 and VP3.

Minipreparations of pBS-PyI-VP1_only plasmid were tested by double digestion with restriction enzymes XhoI and EcoRV in R buffer.

Viral genomes were prepared from plasmids pMJG and pMJ-VP1 with EcoRI enzyme in its specific buffer. Viral genomes prepared from pBS-PyI and pBS-PyI-VP1_only were double digested with SalI and XhoI enzymes in Orange buffer.

4.3.2.5 Dephosphorylation

Mutated VP2/3 genes (insert) and pBS-PyI backbone (vector) were isolated from agarose gel electrophoresis (chapter 4.3.2.1.7) and vector was dephosphorylated with kit Roche Rapid DNA Dephos and Ligation Kit according to manufacturer´s instructions.

4.3.2.6 Ligation

Vector (pBS-PyI) and insert (mutated VP2 and VP3) were ligated in molar ratio 1:3. Total 100 ng of vector was ligated with 300 ng of insert in 30 µl of ligation mixture. For ligation was used 5U of T4 DNA ligase (Thermo Scientific) in T4 DNA ligase buffer. Ligation was incubated over night at room temperature. Ligated plasmid was concentrated with ethanol precipitation.

Viral genome for transfection was prepared with ligation of cleaved plasmid. Ligation mix contained 45 µg of DNA, 15 U of T4 DNA ligase and buffer in total volume of 14 ml. Ligation was incubated for 18 hours in 16°C. Ligated DNA was concentrated with ethanol precipitation (chapter 4.3.2.1.2).

4.3.2.7 PCR

4.3.2.7.1 “Standard” PCR

PCR was used for production of fragments with thymidine analogues incorporated into DNA. Protocol for production of fragments with thymidine analogue by standard PCR was inspired with Tabibzadeh et al. (1991) and Gierlich et al. (2007).

PCR mixture composition:

Polymerase	2 U for Vent, DreamTaq or Deep Vent exo$^-$
Primer 1	0.3 or 0.4 µM
Primer 2	0.3 or 0.4 µM
Specific buffer to polymerase	1x ($MgCl_2$ is included)
dNTPs (A,C,G, EdU/BrdU)	0.2 mM
Template	50 or 400 ng
water (PCR grade)	complemented to final volume 25 or 50 µl

PCR programme:

Initial Denaturation	95°C	3 minutes	
Denaturation	94°C	20 second	
Annealing	57 - 60°C	30 second	repeated 30 x
Elongation	72°C	30 second	
Final Elongation	72°C	5 minutes	
Cooling	4°C		

4.3.2.7.2 Modified PCR for EdU incorporation

This PCR was used to produce fragments with thymidine analogues incorporated into DNA. The modified programme for PCR was taken from Gierlich et al. (2007).

PCR mix composition:

Polymerase	2 U for Vent, DreamTaq or Deep Vent exo$^-$; 1 U for Pwo
Primer 1	0.3 µM
Primer 2	0.3 µM
Specific buffer to polymerase	1x ($MgCl_2$ is included)
dNTPs (A,G,C, EdU)	0.2 mM
TTP	0.02 mM
Template	50 or 400 ng
water (PCR grade)	complemented to final volume 25 µl

PCR programme:

Initial Denaturation	95°C	2 minutes	
Denaturation	95°C	30 second	
Annealing	58°C*	30 second	repeated 7 x
Elongation	72°C	45 second	
Denaturation	95°C	30 second	
Annealing	57°C	30 second	repeated 30 x
Elongation	72°C	45 second	
Final Elongation	72°C	5 minutes	
Cooling	4°C		

*Each cycle the annealing temperature decrease by 1°C

4.3.2.7.3 qPCR

Quantitative PCR was used to determine number of viral DNA copies in sample. DNA was quantified via SYBR Green incorporation into DNA helix. iQ SYBR Green Supermix (Bio-Rad) for PCR is 2x concentrated and it contains antibody-mediated hot-start iTaq DNA polymerase, buffer, dNTPs, 6 mM $MgCl_2$, SYBR Green I, fluorescein and stabilizers. As standard curve for quantification was used pMJG plasmid in decimal dilutions.

PCR mix composition:

iQ SYBR Green Supermix	5 µl (10 µl)
Primer 1	0.5 µl (1 µl) (0.5 µM)
Primer 2	0.5 µl (1 µl) (0.5 µM)
Template	concentration vary with sample
water (PCR grade)	complemented to final volume 10 µl (20 µl)

PCR programme:

Pre-incubation	95°C	3 minutes	
Amplification	95°C	10 second	
	59°C	20 second	repeated 40 x
	72°C	30 second	
Melting curve	95°C	10 second	
	67°C	1 min	
	97°C	not-defined	
Cooling	40°C	45 second	

4.3.2.8 Sequencing

Sequencing was done by Laboratory of DNA Sequencing, Faculty of Science, Charles University in Prague. Laboratory is equipped with 3130 Genetic Analyzer, 3130xl Genetic Analyzer and 3500 Genetic Analyzer (Applied Biosystems). Samples were prepared according to instruction of Laboratory of DNA Sequencing: 3-5 ng of DNA per 100 bp (max. 300 ng of DNA/reaction); 5 pmol of primer; complemented with water (PCR grade) to final volume 8 µl.

4.3.2.9 DNA immunodot-blot assay

DNA was denaturated before "dotting" " (applied as drops) on nylon membrane. DNA was denaturated by incubation at 95°C for 5 minutes and rapidly chilled on ice-water bath for 10 minutes. DNA was centrifuged at 20,000 g for 3 minutes at 4°C and applied on ice on the nylon membrane placed on Whatman paper soaked in PBS. DNA was cross-linked with UV of automatic programme in UV cross-linker from both sides (Gemini B.V.). Membrane was blocked in 5% of low-fat milk for 1 hour on the rocker at room temperature or overnight at 4°C and 20 minutes at room temperature on the rocker. Blocked membrane was sealed in a foil with 1 ml of primary antibody diluted in 5% of low-fat milk and incubated for 1 hour at room temperature on the rocker or overnight at 4°C. Membrane was washed in PBS 3 times for 10 minutes at room temperature on the rocker. After washing, membrane was sealed with secondary antibody conjugated with HRP diluted in 5% low-fat milk. Membrane was again washed in PBS 3 times for 10 minutes at room temperature on rocker. Membrane was incubated for 30 seconds in luminol solution (mixed solution A and B) and medical X-ray film blue (Agfa) was exposed to signal on the membrane. X-ray film was developed, fixed and washed in ample of water.

4.3.2.10 DNA hybridization

DNA hybridization was performed with DNA samples isolated from virus inoculum in hybridization oven. Hybridization was performed with DIG High Prime DNA Labelling and Detection kit II (Roche) according to manufacturer´s instruction. Probe was made by Lenka Horníková from plasmid with viral DNA (pMJG) fragments. In brief, DNA samples were "dotted" on nylon membrane placed on Whatman paper soaked in 10x SSC buffer (1.5 M NaCl; 0.15 M sodium citrate; pH = 7). DNA on membrane was cross-linked in UV cross-linker on automatic programme from both sides. Membrane was pre-hybridized in DIG Easy Hyb. buffer

solution for 30 minutes at 43°C in hybridization oven. Solution was removed and 1 ml of the probe in DIG Easy Hyb. buffer (25 ng/ml) was applied on membrane and incubated in hybridization oven overnight at 43°C. Membrane was washed 2 times for 5 minutes with 2x SSC buffer with 0.1% SDS at room temperature on the rocker. Membrane was washed again 2 times for 15 minutes with 0.5x SSC with 0.1% SDS buffer at 68°C in hybridization oven. After washing, membrane was blocked in blocking solution for 1 hour at room temperature. Blocked membrane was incubated with primary antibody (αDIG) for 1 hour at room temperature on rocker. Membrane was washed 2 times for 15 minutes in washing solution on rocker. After washing, membrane was incubated in detection buffer for 5 minutes at room temperature. Detection buffer was removed and membrane was incubated in CSPD ready-to-use solution for 5 minutes. Medical X-ray film Blue (Agfa) was exposed to the signal on membrane. Film was developed, fixed and washed in ample of water.

4.3.3 Work with bacteria

4.3.3.1 Cultivation for plasmid production

For the production of all plasmids were used monocolonies of bacterial culture spread on nutrient agar plate with appropriate antibiotics. Monocolony was inoculated into LB media with appropriate antibiotics and incubated for 12 - 16 hours on shaker at 200 r.p.m., 37°C.
Bacteria expressing low copy plasmids pMJG and pMJ-VP1 were inoculated into 5 ml of LB media with appropriate antibiotics and incubated (shaking at 200 r.p.m., 37°C) 12 - 16 hours. Then 0.2 ml of this bacterial culture was inoculated into 100 ml of LB media and incubated with appropriate antibiotics (200 r.p.m., 37°C) for 7 hours. After 7 hours of incubation chloramphenicol was added to the culture to final concentration 170 μg/ml and continuation incubation for another 12 - 16 hours.

4.3.3.2 Preparation of competent cells

XL-1 Blue bacteria were spread on nutrient agar plate and incubated at 37°C overnight. A monocolony from nutrient agar plate was inoculated into 10 ml of TPN broth and incubated with shaking (200 r.p.m.) overnight at 37°C. Next day, 400 ml of TPN broth was inoculated with the overnight culture to final OD_{560} 0.1. Inoculated broth was incubated at 37°C with shaking (200 r.p.m.) till OD_{560} 0.5-0.7 (mid-log growth phase). The culture was briefly chilled down on

ice after incubation and centrifuged at 2000 g for 10 minutes at 4°C. Bacteria were resuspended in 400 ml of sterile cold dH_2O and centrifuged as above conditions. Bacteria were resuspended in 200 ml of sterile cold dH_2O and centrifuged as above conditions. Bacteria were resuspended in 16 ml of sterile cold 10% glycerol (w/w) and centrifuged as above conditions. This step was repeated with resuspension in 8 ml of sterile cold 10% glycerol (w/w). Finally, bacteria were resuspended in 0.8 ml of sterile cold 10% glycerol (w/w) and aliquoted (50 µl, 100 µl or 150 µl) in microtubes. Bacteria were frozen in liquid nitrogen and stored at -80°C.

4.3.3.3 Bacteria transformation by electrophoresis

For one electroporation, 50 µl of competent bacteria were mixed with 1 µl of plasmid. Mixture was transferred to electroporation cuvette (Bio-Rad) with interelectrode distance 2 mm. The electroporation pulse had capacitance 25 µF, voltage 2.5 kV, resistance 200 Ω and last for 4.5 - 5 miliseconds. After the pulse was applied, 1 ml of SOC media was added to cuvette and suspension was transferred to Erlenmayer flask. Suspension was incubated at 37°C with shaking (200 r.p.m.) for 1 hour. After incubation, variety of volumes (1 µl, 10 µl, 100 µl and the rest of culture) of bacteria were spread on nutrient agar plates and incubated overnight at 37°C.

4.3.3.4 Minipreparation of plasmids

Monocolonies from nutrient agar plate were inoculated in 0.7 ml of nutrient broth with appropriate antibiotics in microtubes and incubated overnight with shaking (200 r.p.m.) at 37°C. Plasmids from bacteria were isolated according to the chapter 4.3.2.1.9.

4.3.3.5 Bacterial conserves

Bacteria were cultivated in nutrient broth overnight with shaking (200 r.p.m.) at 37°C. Overnight culture (0.7 ml) was transferred in microtube and 50% (w/w) glycerol (0.3 ml) was added. Bacteria were frozen in liquid nitrogen and stored at -80°C.

4.3.4 Virus preparation

4.3.4.1 Virus production

For virus preparation, 3T6 cells were passaged 1:6 on ∅ 10 cm dish. Day after passaging (confluence of cells ~ 60%), cells were infected with multiplicity of infection (MOI) = 0.1 for production of wild-type virus or MOI = 5 for wild-type virus with EdU/BrdU incorporated in DNA. Cells were washed with serum free DMEM medium, 1 ml of serum free DMEM medium with virus was applied on cells and cells were incubated for 90 minutes in 37°C, 5% CO_2 with gentle rocking every 20 minutes. After incubation, 9 ml of DMEM serum supplemented with antibiotics was added to the cells. Cells were incubated in 37°C, 5% CO_2 for 7 to 10 days (depending on progress of infection) for wild-type virus. Cells were frozen in -80°C after incubation.

EdU/BrdU to production was added 20 hours post infection to final concentration 1 μg/ml (4 respectively 3.2 μM). The same dose was added again 4 days post infection. Virus was harvested 5 - 6 days post infection.

4.3.4.2 Mutant virus production

Non-infectious virus was produced by transfection (see chapter 4.3.5.4). Transfected cells were incubated for 3 - 4 days and then the virus was isolated (when cells reached confluence). Genome of mutated virus was produced *in vitro* from plasmid pMJ-VP1 or plasmid pBS-PyI-VP1_only.

4.3.4.3 Virus isolation

Dishes with infected cells were 3 times froze-thawed. Cells were scrubbed and transferred to 50 ml tube with media and centrifuged at 8,000 g for 30 minutes at 4°C. Supernatant was stored at 4°C and pellet was resuspended in 1 ml of B-buffer. Suspension was homogenized in Potter´s homogenizer on ice. Homogenized suspension was transferred to 50 ml tube and aprotinin (final concentration 2 μg/ml) and neuraminidase (final concentration ~0.02 U/ml) was added. Suspension was incubated at room temperature on rocker for 16 hours. Suspension was centrifuged at 6,000 g for 30 minutes at 4°C. Supernatant was added to

previous one and pellet was resuspended in 1 ml Tris-HCl pH = 9. Suspension was incubated at room temperature on rocker for 4 hours and centrifuged at 8,000 g for 30 minutes at 4°C. Supernatant was mixed with previous one and pellet was discarded. Mixed supernatants were centrifuged through 10% sucrose cushion in ultracentrifuge at 25,000 r.p.m. for 3 hours at 4°C (rotor SW28). Supernatant was discarded and pellet was slowly dissolved (overnight at 4°C) in 1 ml of B-buffer. Virus suspension was centrifuged in caesium chloride gradient at 35,000 r.p.m. for 24 hours at 18°C (rotor SW41 or SW 55Ti). Initial refraction index (density of suspension) was 1.364 - 1.366. Gradient was separated into fractions using fraction recovery system (Beckman). Refraction index was measured from each fraction and dot-blot (VP1 detection) was performed to analyze fractions. After fraction analysis, a dialysis was performed. Fractions were combined to three fractions (based on refraction index and VP1 location on dot-blot). Dialysis tubing (Serva) was boiled for 10 minutes in dH_2O and filled with virus suspension. Each fraction of virus was closed with clips from both sides. Dialysis was performed in 2.5 litres of B-buffer at 4°C on magnetic stirrer. After 1 hour, B-buffer was changed for fresh one and dialysis continued overnight. Dialysed virus was centrifuged through 10% sucrose cushion in ultracentrifuge at 35,000 r.p.m. for 3 hours at 4°C (rotor SW41). Pellet was slowly dissolved (overnight) in B-buffer (volume depends on quantity of virus) and transferred in low retention microtubes.

EdU virus and BrdU virus were treated with DNase I after isolation. Viral inoculum was mixed with $MgCl_2$ (final concentration = 10 mM) and 40 U of DNase I. Virus was incubated for 1 hour at 37°C. DNase I was removed by centrifugation (rotor SW 55Ti, 35000 r.p.m., 4°C, 127 minutes) through 10% sucrose cushion 2 times.

4.3.4.4 Infection for indirect immunofluorescence

3T6 cells were washed with Versen solution and trypsinized. Cell were resuspended in 6 ml of serum supplemented DMEM passaged on 24-well plate. 30 µl of cell suspension were passaged on one well. 50 µl of cell suspension were passaged as duplicate. A day after passaging, cells were washed with serum-free DMEM medium and infected with viral inoculum or with 0.05 ml of medium with lysed cells diluted in 0.2 ml of serum-free DMEM medium. Cells were incubated for 90 minutes at 37°C, 5% CO_2 with gently rocking every 20 minutes. 1 ml of serum supplemented DMEM medium with antibiotics was added to infected cells. Cells were cultivated for variety of times.

4.3.4.5 Determination of viral titre

3T6 cells were seeded on cover slips in 24-well plate. Next day, cells were infected with series of decimal dilution of virus. 24 hours post infection were cells fixed and indirect immunofluorescence was performed with primary antibody rat αLT1 in dilution 1:100 and secondary antibody αrat Alexa Fluor 488 conjugate (dilution 1:1000). Cells positive on LT antigen were counted and viral titre was calculated.

4.3.5 Work with tissue culture

All work with tissue culture was done with mammalian cells which require pre-warmed media, 0.25% trypsin in PBS, Versen solution (0.02% EDTA in PBS). Cells were cultivated at 37°C in 5% CO_2 atmosphere. Maintenance passaging was done with serum supplemented DMEM medium without antibiotics.

4.3.5.1 Passaging of cells

Medium was removed from dishes with confluent cells and cells were washed with Versen solution. Cells were trypsinized (0.5 ml per ∅ 6 cm dish, 1 ml per ∅ 10 cm dish) at 37°C, 5% CO_2 until cells detached from surface (max. 10 minutes). Cells were resuspended in serum supplemented DMEM medium and passaged on fresh dishes with fresh media (total volume for ∅ 6 cm dish is 6 ml of media and for ∅ 10 cm dish is 10 ml of media).

4.3.5.2 Transfection with cationic polymer delivery

Transfection was performed TurboFect Transfection Reagent (Thermo Scientific). Cells were passaged and transfected according to manufacturer´s instructions.

4.3.5.3 Transfection with electroporation

Cells were passaged 1:5 on ∅ 10 cm dish. Second day (cell confluence ~70%), cells were transfected with electroporation using Amaxa nucleofector. Cells were trypsinized, resuspended in 5 ml per dish of serum supplemented DMEM and counted in Bürker cell counter. Volume with 4×10^6 cells per one reaction was transferred in the fresh centrifugation

tube and centrifuged at 90 g for 10 minutes at room temperature. Medium was removed and 100 µl per reaction of Nucleofector V solution (Lonza) was added to cells in which they were resuspended. Solution was mixed with 6 µg of plasmid or 4 µ of ligation solution containing viral genome. Solution with DNA was transferred into electroporation cuvette with interelectrode distance 2 mm. Pulse was applied with pre-set programme for cells (T-030 for 3T6 and T2 cell lines or U-030 for 3T3 cell line). 1640 RPMI medium was added to transfected cells (0.5 ml) and suspension was transferred to microtube. Cell suspension was incubated at 37°C, 5% CO_2 for 15 minutes and afterwards the suspension was transferred into serum supplemented DMEM medium with antibiotics on dish or 24-well plate and cultivated. Medium was replaced after 3 - 4 hours.

4.3.6 Hemagglutination assay

Guinea pig red blood cell was washed from conservation solution with 0.2% BSA with PBS (washing of blood was done by technician Ivana Polívková). Red blood cells were centrifuged at 700 g for 20 minutes at 4°C and 3 times washed with 0.2% BSA in PBS and. Concentration of red blood cells is determined via hematocrit. Red blood cells suspension was carefully mixed and 30 µl of suspension was transferred to heparinised 75 mm capillary. The capillary is sealed on both sides with flame. Capillary is centrifuged for 5 minutes at 2,500 r.p.m. (centrifuge MPW 300 with hematocrit rotor). After centrifugation, hematocrit is determined as percent of PBS column height.

Working concentration of red blood cells is 0.4% in 0.2% BSA in PBS. Assay was performed in 96-well plate with round bottom. Viral inoculum was diluted in 0.2% BSA in PBS to final volume 50 µl. Three dilutions were prepared from sample - 10x dilution, 100x dilution and 1000x dilution. Wells in the plate were filled with 50 µl of 0.2% BSA in PBS. First well of each line was mixed 50 µl of different dilutions of samples with 50 µl of 0.2% BSA in PBS. In each line, binary dilution series was made. Dilutions were mixed with 50 µl of 0.4% red blood cells and plate was incubated for 1 hour at room temperature or overnight at 4°C. Hemagglutination units (HAU) were determined from assay and number of particles per millilitre was calculated using following equation (empirically determined).

$$HAU \cdot dilution \cdot 10^7 = number\ of\ particles/ml$$

4.3.7 Work with proteins

4.3.7.1 Concentration determination

Concentration of protein in sample was measured with Qubit fluorometer (Invitrogen) using Protein Assay Kit (Invitrogen).

4.3.7.2 Immunodot-blot assay

Protein dot-blot was used to verify presence of VP1 in fraction gathered from Cesium Chloride gradient. 4 µl of each fraction was "dotted" on nitrocellulose membrane. Membrane was blocked in 5% of low-fat milk for 1 hour on rocker at room temperature or overnight at 4°C and 20 minutes at room temperature on rocker. Blocked membrane was sealed in foil with 1 ml of primary antibody diluted in 5% of low-fat milk and incubated for 1 hour at room temperature on rocker or overnight at 4°C. Membrane was washed in PBS 3 times for 10 minutes at room temperature on rocker. After washing, membrane was sealed with 1 ml secondary antibody conjugated with HRP diluted in 5% low-fat milk and incubated for 0.5 hour at room temperature on rocker. Membrane was again washed in PBS 3 times for 10 minutes at room temperature on rocker. Membrane was incubated for 30 seconds in luminol solution (mixed solution A and B) and medical X-ray film blue (Agfa) was exposed to signal on the membrane. X-ray film was developed, fixed and washed in large amount of water.

4.3.7.3 Western-blot

4.3.7.3.1 Sample preparation

Virus inoculum (3 µl) was diluted in 37 µl RIPA buffer (150 mM NaCl; 5 mM EDTA; 50 mM Tris-HCl pH = 7.4; 0.05% NP-40; 1% sodium deoxycholate; 1% Triton X-100; 0.1% SDS) with protease inhibitor cocktail (Roche).

Cells on ∅ 6 cm dish were scraped and resuspended in media. 1 ml of suspension was transferred into microtube and centrifuged at 20,000 g for 30 seconds. Pellet was resuspended in 80 µl of RIPA buffer with protease inhibitor cocktail (Roche).

Samples in RIPA buffer were incubated for 20 minutes on ice. To lysed samples was added 5x Laemmli sample buffer (5% SDS; 50 mM Tris-HCl pH 6.8; 50 % (V/V) glycerol; 25% (V/V) β-mercaptoethanol; 0.005 % (w/V) bromphenol blue) to final concentration 1x. Samples were incubated for 5 minutes at 100°C and centrifuged at 20,000 g for 1 minute. Samples were stored at -20°C.

4.3.7.3.2 SDS-PAGE

SDS-PAGE was used for separation of proteins in cell or viral lysate by molecular weight. Apparatus for gel preparation was assembled according to manufacturer´s instruction. Tightness of apparatus was checked with water. Apparatus was perfectly dried. Lower gel was poured to the apparatus (~ 1 cm below the end of comb) and overlaid with ddH_2O. Lower gel was left for polymerization for about 30 minutes (polymerization was checked with gel left in tube). Upper gel was poured on the lower gel, comb was inserted in apparatus and gel left for polymerization. Gel was transferred to electrophoresis apparatus and overlaid with running buffer (25 mM Tris; 192 mM glycine; 0.1% (w/V) SDS). Sample holes were washed with running buffer and samples were loaded (10 - 25 µl) alongside with 10 µl of marker. Electrophoresis ran for 30 minutes (through upper gel) at voltage 80 V and afterward at voltage 140 V till the bromphenol blue reached end of the gel.

4.3.7.3.3 Western-blot

When proteins were separated on the gel, they were transferred on nitrocellulose membrane by western-blotting. Gel was incubated for 10 minutes in blotting buffer (25 mM Tris; 195 mM; 20% (V/V) methanol). Blotting sandwich was assembled (sponge, 4x filter paper, Whatman paper, membrane, gel, Whatman paper, 4x filter paper, sponge) and placed in blotting apparatus. Transfer was performed at 250 mA for 3 hours.

4.3.7.3.4 Detection

Membrane with transferred proteins was blocked in 5% low-fat milk for 1 hour at room temperature on rocker or overnight at 4°C and 20 minutes at room temperature on rocker. Blocked membrane was sealed in foil with 1 ml of primary antibody diluted in 5% low-fat milk and incubated for 1 hour at room temperature on rocker or overnight at 4°C and 20 minutes at room temperature on rocker. Membrane was washed in PBS 3 times for 10 minutes on rocker.

Membrane was sealed in foil with secondary antibody conjugated with HRP diluted in 5% low-fat milk and incubated for 30 minutes at room temperature on rocker. Membrane was washed in PBS 3 times for 10 minutes on rocker. After washing, membrane was incubated in luminol solution for 30 seconds. Medical X-ray film Blue (Agfa) was exposed to signal on membrane. Film was developed, fixed and washed in ample of water.

4.3.8 Immunofluorescence

4.3.8.1 Indirect immunofluorescence

Cells grown on cover slip were washed with PBS and fixed with 3.7% (w/V) formaldehyde for 15 minutes. Fixed cells were rinsed in PBS and permeabilized for 5 minutes in 0.5% (V/V) Triton X-100. Cells were 3 times rinsed in PBS and blocked in blocking solution 1 for 30 minutes. After blocking, primary antibody diluted in blocking solution 1 was applied on the cells for 1 hour at room temperature or overnight at 4°C. Cells were washed 3 times in PBS for 10 minutes and secondary antibody diluted in blocking solution 1 was applied on cells for 30 minutes. Cells were washed again 3 times in PBS for 10 minutes. Cells were rinsed in ddH_2O and allowed to air-dry. Dried cells were mounted in DAPI solution in 50% glycerol (w/w). All incubations were at room temperature unless otherwise stated.

4.3.8.2 Optimization of indirect immunofluorescence for BrdU detection

Detection of BrdU with antibody is problematic. If we want to detect BrdU in genome we need to denature DNA (usually with DNase or HCl) to make BrdU accessible to antibody. Method for detection of BrdU wasn´t established in our lab before. Detection of BrdU and second antigen is not an easy task so we have employed 3 methods of BrdU detection. Cells were pseudo infected with viral particles (chapter 4.3.4.4) and treated with 1 μg/ml of BrdU. Cells were fixed 24 hours post pseudo infection and following protocols were applied.

4.3.8.2.1 BrdU staining with HCl treatment

This protocol was acquired from Springer Lab Manual chapter (Cardoso and Leonhardt, 1995).

1. Cells were rinsed with PBS 2 times
2. Fixation with 3.7% formaldehyde for 15 minutes at, rinsed 3 times with PBS
3. Permeabilization with 0.5 % for 5 minutes, rinsed 3 times with PBS
4. Blocking in blocking solution 1 for 30 minutes
5. Added first antibody diluted in blocking solution 1 for 1 hour, rinsed 3 times with PBS containing 0.1% NP-40
6. Added secondary antibody diluted in blocking solution 1 for 30 minutes, rinsed 3 times with PBS containing 0.1% NP-40
7. Cell were fixed again as described in step 2
8. Treated 4 N HCl with 1% Triton X-100 for 10 minutes, rinsed 3 times with PBS containing 0.1% NP-40
9. Added BrdU antibody diluted in blocking solution 1 for 1 hour, rinsed 3 times with PBS containing 0.1% NP-40
10. Added antibody against BrdU antibody diluted in blocking solution 1 for 1 hour, rinsed 3 times with PBS containing 0.1% NP-40
11. Cells were rinsed in ddH_2O and allowed to air-dry
12. Cells mounted in DAPI solution in 50% glycerol (w/w)

Protocol was performed at room temperature.

4.3.8.2.2 BrdU staining with DNase I treatment

This protocol was obtained from Springer Lab Manual chapter (Cardoso and Leonhardt, 1995).

1. Cells were rinsed with PBS
2. Fixation with 3.7% formaldehyde for 15 minutes at, rinsed 3 times with PBS
3. Permeabilization with 0.5 % for 10 minutes, rinsed 3 times with PBS
4. Blocking in blocking solution 1 for 30 minutes
5. Added first antibody and BrdU antibody diluted in DNase buffer solution (0.5 mM β-Mercaptoethanol; 30 mM Tris-HCl pH = 8.1; 0.33 mM $MgCl_2$) with DNase I (2 U per one coverslip) and 1% BSA in PBS, rinsed 3 times with PBS
6. Added secondary antibodies diluted in blocking solution 1 for 30 minutes, rinsed 3 times with PBS
7. Cells were rinsed in ddH_2O and allowed to air-dry
8. Cells mounted in DAPI solution in 50% glycerol (w/w)

Protocol was performed at room temperature.

4.3.8.2.3 Protocol from BrdU antibody manufacturer (Abcam)

1. Fixation and permeabilization with chilled (-20°C) methanol (100%) for 5 minutes at -20°C
2. Methanol was removed and cell were allowed to air-dry
3. Incubation for 30 minutes at room temperature in 2 M HCl diluted in PBS with 0.1% Tween
4. Neutralization on 100 µl drop of 1% BSA (w/V), 10% (V/V) FBS, 0.3 M glycine in PBS with 0.1% Tween for 1 hour at room temperature
5. Added primary antibody with BrdU antibody overnight at 4°C, washed 3 times for 10 minutes in PBS with 0.1% Tween
6. 6. Added secondary antibodies diluted in 1% BSA in PBS for 30 minutes, washed 3 times for 10 minutes in PBS with 0.1% Tween
7. Cells were rinsed in ddH_2O and allowed to air-dry
8. Cells mounted in DAPI solution in 50% glycerol (w/w)

4.3.8.2.4 Optimization

None of the above protocols gave us satisfactory results. Best result for detection of BrdU is with DNase protocol (4.3.7.2.2) but second detection was ruined. Best result for detection of VP1 gave us first protocol with HCl denaturation. For optimization we chose to combine these two protocols. After a few attempts we developed following protocol.

1. Cells were rinsed with PBS
2. Fixation with 3.7% formaldehyde for 15 minutes at, rinsed with PBS
3. Permeabilization with 0.5 % for 10 minutes, rinsed 3 times with PBS
4. Blocking in blocking solution 2 for 30 minutes
5. Added first antibody diluted in blocking solution 2 for 1 hour, washed 3 times for 10 minutes in PBS
6. Added secondary antibody diluted in blocking solution 2 for 30 minutes, washed 3 times for 10 minutes in PBS
7. Cell were fixed again as described in step 2
8. Added BrdU antibody diluted in DNase buffer solution without β-Mercaptoethanol with DNase I and 5% teleostean gelatine in PBS, washed 3 times for 10 minutes in PBS
9. Added secondary antibody diluted in blocking solution 2 for 1 hour, washed 3 times for 10 minutes in PBS
11. Cells were rinsed in ddH_2O and allowed to air-dry
12. Cells mounted in DAPI solution in 50% glycerol (w/w)

4.3.9 Click chemistry

We used Click chemistry to detect DNA with incorporated nucleotide - alkynes (specifically EdU). Azide is covalently bonded to alkyne in highly specific manner. To detect EdU in cells we used EdU Imaging Kit (Molecular Probes) with Alexa Fluor - azide 594. Detection was performed strictly according to manufacturer´s instruction. Indirect immunofluorescence can be performed after click reaction.

We used Click chemistry also in solution with fragment of pMJG produced by PCR with EdU analogue. We applied 2 protocols for click chemistry but both of them didn´t provide us with satisfactory results. Composition of Click reaction is summarized in table 4.1. Reaction was incubated for 4 hours at room temperature. DNA labelled with Alexa Flour Azide was

analyzed in DNA electrophoresis before and after purification of DNA with commercial kit (NucleoSpin Gel and PCR Clean-up kit from Macherey - Nagel according to manufacturer´s instruction). Agarose gel for DNA electrophoresis was prepared without EtBr. DNA in gel was stained after electrophoresis in TAE/TBE buffer bath with 0.5 µg/ml EtBr bath for 15 minutes.

1. Method		2. Method	
Solution	Final concentration (mM)	Solution	Final concentration (mM)
DNA Alkyne	0.064	water	-
water	-	$CuSO_4$ + THPTA (1:5)	0.5 + 2.5
Alexa Fluor Azide	~0.13	Sodium Ascorbate	5
$CuSO_4$ + THPTA (1:5)	0.5 + 2.5	HEPES	40
Aminoguanidin-HCl	5	NaCl	250
Sodium Ascorbate	5	DNA Alkyne	0.08
Total volume	50 µl	Alexa Fluor Azide	~0.16
		total volume	50 µl

Table 4.1: Table summarize solutions and their concentration in click reaction. DNA alkyne is PCR product which should have incorporated EdU instead of Thymidine (see chapter 4.3.2.7.3). All solutions were added to reaction in exact order as described in table.

4.3.10 Negative staining for electron microscopy

5 µl of viral inoculum was transferred on parafilm as drop. Copper or Nickel grid with carbon coated formvar membrane was placed on the drop for 10 minutes. Grid was washed 2x on 100 µl drops of ddH_2O for 30 seconds and contrasted on of 50 µl drops of 2% phosphotungstic acid (pH = 7) for 30 second (2 x). Phosphotungstic acid was carefully removed and grid was dried at room temperature.

4.3.11 Preparation of samples for electron microscopy

4.3.11.1 Embedding in Agar 100

3T6 cells were transfected with viral genomes produced from pMJG or pMJ-VP1 plasmids with Amaxa nuclefection system. Cells were washed in PBS and fixed 48 h.p.i. with 3% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate in PBS, pH = 7.5) at 4°C for 1 hour. After 30 minutes of fixation, cells were scrubbed, transferred to the microtube and used fixative was replaced with fresh one. Scrubbed cells were transferred in microtube. Cells were washed in cacodylate buffer 2 times for 5 minutes. Cells were fixed with 1% OsO_4 in cacodylate buffer at 4°C for 1 hour. After fixations, cells were washed in cacodylate buffer 2 times for 5 minutes. Buffer was removed and fixed cells were mixed with 0.5 ml of pre-warmed 10% porcine gelatine and incubated in warm bath (37°C) for ten minutes with occasional stirring. Cell were centrifuged at 1.5 rcf for 5 minutes and placed in iced bath for 30 minutes. Hardened gelatine with cells was cut into cubes (approx. 1 mm^3). Cubes were dehydrated in ethanol series - 30%, 50%, 70%, 90%, 95%, 100% and 100% for 15 minutes in each dilution. After dehydration cubes were incubated in propylene oxide 2 times for 10 minutes. Cubes were transferred in mixture of propylene oxide and agar 100 in ration 2:1 for 15 minutes. Same step was repeated with mixture in ration 1:1 and 1:2. Next, cubes were transferred to pure agar 100 and incubated at 4°C overnight. Next day, cubes were incubated at room temperature for 1 hour. Used agar 100 was removed after incubation and fresh one was added for 3 hour. Cubes were after incubation encapsulated in gelatine capsules in which cubes were polymerized at 60°C for 72 hours. After polymerization, cells were cut on microtome to ultrathin sections.

4.3.11.2 Embedding in LR White

3T6 cells were transfected as described in the previous chapter. Transfected cells were washed with in PBS and fixed with 4% PFA and 0.05% glutaraldehyde in SB buffer (Sörenson´s buffer - 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4, pH 7.3) on ice for 1 hour. Fixed cells were washed on ice with SB buffer two times for 10 minute. Cells were scrubbed, transferred into microtube and mixed with 10% porcine gelatine as described in previous chapter. Cells in gelatine were incubated in ice bath for 1 hour and hardened cells in gelatine were cut into cubes (1 mm^3). Cubes were dehydrated on ice in ethanol series - 30%, 50%, 70%, 90% and 96% for 10 minutes in each dilution. Cubes were then incubated in LR white 96% ethanol mixture in ration 1:2 for

20 minutes on ice. Same step was repeated with mixture in ration 1:2. Next, cubes were incubated in pure LR white on ice for 2 hours and then overnight with fresh LR white at 4°C. Next day, cubes were incubated in fresh LR white for 4 hours at 4°C. Cubes were encapsulated and LR white was polymerized under UV light at 4°C for 72 hours. Polymerization continued for 3 hours at room temperature. Polymerized cells were cut in ultrathin sections. Sections on nickel grid were labelled with antibodies. Sections were rehydrated with dH_2O for 10 minutes and blocked in 10% normal goat serum with 1% BSA in PBS for 30 minutes. Primary antibody diluted 1:10 - 1:50 (depends on antibody) in 0.5% BSA with 0.1% teleostean gelatine was applied for 1 hour at room temperature or overnight at 4°C. Primary antibody was washed with 0.1% BSA in PBS 3 times for 10 minutes. Secondary antibody diluted 1:20 in 0.5% BSA with 0.1% teleostean gelatine in PBS was applied for 50 minutes at room temperature. Secondary antibody was washed with 0.1% teleostean gelatine in PBS 2 times for 10 minutes. Attached antibodies were fixed with 1% glutaraldehyde in PBS for 10 minutes and washed 2 times for 10 minutes in dH_2O. Sections on grid were air dried and contrasted with 3% uranyl acetate in dH_2O for 10 minutes. Uranyl acetate was through vigorous stirring of the grid in dH_2O for 2 minutes and again in fresh dH_2O for 1 minute. Grids were air dried.

4.3.11.3 High Pressure Freezing and Freeze substitution

High pressure freezing and freeze substitution was performed with help of Pavel Jůda and Jana Šmigová from First Faculty of Medicine according to their protocol published in (Smigova et al., 2011). 3T6 cells were seeded on sapphire discs and infected with wild-type virus with MOI = 1000. Monolayer of cells was fixed 4 h.p.i. by high pressure freezing (Leica PACT2) on sapphire disc. Rest of the infected cells were scrubbed, transferred into specimen holder and fixed by high pressure freezing. Frozen samples were transferred into automatic freeze substitution apparatus (Leica EM AFS2) and samples were subtitled for Lowicryl HM20. Cells embedded in Lowicryl (HM20) were cut into ultrathin section. Sections on grids were labelled as described in previous chapter.

5. Results

5.1 Production and characterization of the VP1_only viral mutant

Series of MPyV genomes with different mutations in genes encoding VP2 and VP3 proteins have been constructed in the backbone of derivative of pBR322 plasmid in our lab over the years (Bilkova, 2013; Cibulka, 2013; Huerfano, unpublished data; Mannova et al., 2002). The production of these mutants that are usually non-viable requires the repeated rounds of transfection of large amount of cells with mutant genome. The genome therefore must be prepared in large scale by excision of viral genome from parental plasmid, several fold dilution (to favour molar ratio for correct circulation), re-ligation and subsequent concentration of DNA sample. This process is time consuming and quantity of correctly re-ligated viral genome varies between batches in each ligation mixtures. In contrast, the novel system developed in our laboratory allows easier and faster virus production. The system is based on Cre-LoxP recombination (Hron et al., 2013). Cre-LoxP system of virus production is designed in high-copy plasmid with two LoxP sites introduced on the boundary of viral genome that is autonomously recombined (re-circularized) *in vivo* in Cre recombinase expressing cells (Fig. 5.1). The expression of Cre recombinase is ensured either by co-transfection with plasmid carrying Cre recombinase gene, or by usage of cell line with stable expression of Cre recombinase. Cre recombinase is tyrosine recombinase enzyme which uses topoisomerase I like mechanism for homological recombination between two LoxP sites.

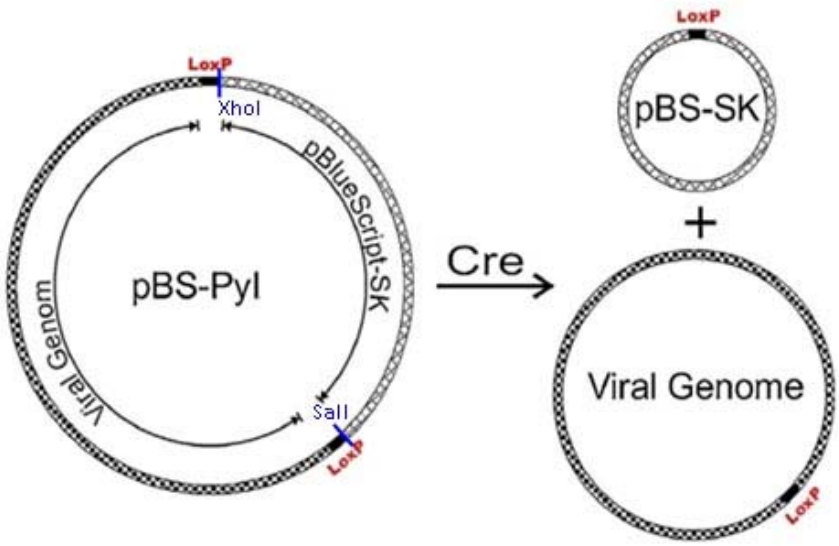


Figure 5.1: Illustration of Cre-LoxP mediated recombination in pBS-PyI plasmid. Cre- recombinase specifically cleaves the plasmid in two LoxP sites and ligates the plasmid backbone and viral genome into circular form each with one LoxP site. The LoxP site in viral genome is located in common intron sequence of T antigenes. Adapted from Hron et al., (2013).

One of the constructs previously prepared in our lab is plasmid pMJ-VP1. This vector contains polyomavirus genome with altered start codons for both, VP2 and VP3. The virus which is produced from this plasmid lacks both minor proteins and is completely non-infectious. Similarly to other mutant viruses with lesion in minor proteins, the reason for reduced infectivity is not completely clear. For SV40, the VP2 and VP3 proteins were shown to be involved in genome encapsidation, but for MPyV no specific encapsidation sequence was found and no specific role for minor proteins was in this process was assigned. Nevertheless, the VP1_only mutant virus was never characterized in respect to its DNA content. Furthermore, the latest results from our lab suggest, that VP2 and VP3 proteins are necessary for correct translocation of VP1 from cytoplasm to nucleus after its synthesis (Lenka Horniková; unpublished results). This phenomenon could therefore influence the production of mutant VP1_only virus.

VP1_only virus was selected for verification of the utility of novel Cre-LoxP system for polyomavirus mutant production and analysis of DNA content and investigation of intracellular formation of virions.

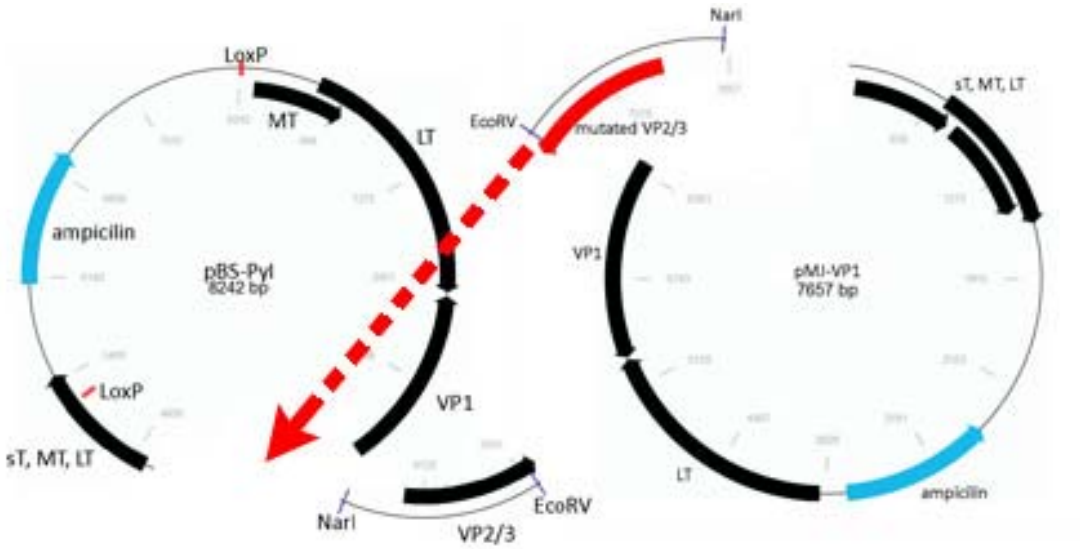


Figure 5.2: Illustration of pBS-PyI plasmid and pMJ-VP1 plasmid with excised VP2 and VP3. Red dashed line represents ligation of excised mutated VP2/3 from pMJ-VP1 into pBS-PyI.

5.1.1 Preparation of mutant genome for Cre-LoxP system

We have decided to place previously mutated VP2 and VP3 sequences from pMJ-VP1 to the pBS-PyI plasmid (Fig. 5.2) in place of wild-type proteins. pBS-PyI and pMJ-VP1 were digested in NarI and EcoRV sites which enclose genes for VP2 and VP3. Then mutated genes from pMJ-VP1 were ligated into digested pBS-PyI with same enzymes. In pMJ-VP1 VP2 is mutated by insertion of stop codon 6 nucleotides after ATG by 4 nucleotide deletion. VP3 is mutated by nucleotide substitution in its start codon.

Prepared plasmid was transfected into bacteria via electroporation. Transformed bacteria were spread on nutrient agar plates and several monocolonies were chosen for analysis. Plasmid minipreparations were tested with restriction enzymes XhoI and EcoRV. EcoRV is one of the restriction sites we used for construction and XhoI is located in the vector. Digestion with these two enzymes resulted in two bands with expected length 6267 bp and 1975 bp. Most of the tested colonies contained desired plasmid (Fig. 5.3)

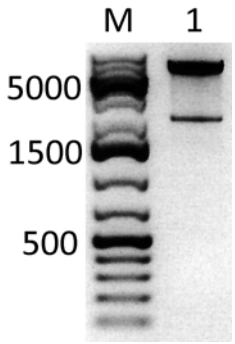


Figure 5.3: Verification of successful cloning of mutated VP2/3 into pBS-PyI. Final construct is digested with Xho and EcoRV enzymes. Size of the bands is 6267 bp and 1975 bp.

After verification with restriction enzymes, 4 plasmids were chosen for sequence analysis. All 4 plasmids contained the desired mutation (Fig.5.4).

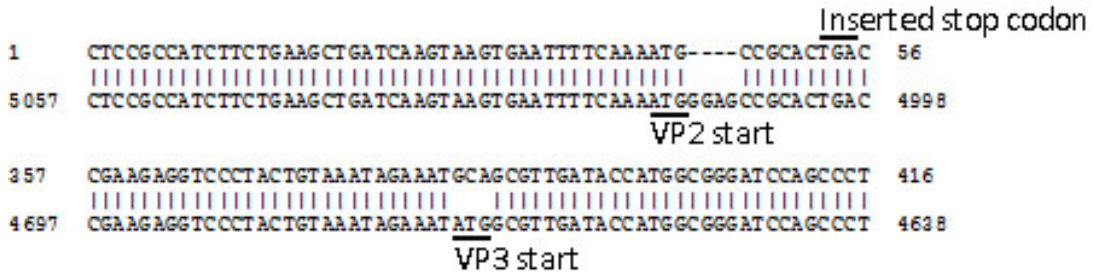


Figure 5.4: Alignment of plasmid pBS-PyI-VP1_only (upper row) and MPyV BG strain (lower row). Start codon of VP2 is in position 5014 and VP3 start codon is in position 4669. VP2 was deleted by insertion of stop codon via frameshift mutation (4 nucleotide deletion behind start codon). VP3 start codon was mutated.

5.1.2 Production of mutant virus from Cre-LoxP system

Transfection efficiency and viral protein production from pBS-PyI-VP1_only vector have been compared to other experimental set-ups by series of transfections. The production from "novel" pBS-PyI-VP1_only system (*in vivo* recombination) was compared with "standard" (*in vitro* excision and re-ligation) method for pBS-PyI-VP1_only vector as well as for pMJ-VP1. Two restriction sites (XhoI and SalI) were added in plasmid pBS-PyI. After digestion with these enzymes, the viral genome is produced with two LoxP sites in the common intron region for T antigens but these LoxP sites do not affect the infectivity of produced wild-type virus (Hron et al., 2013). These LoxP sites remained also in the viral genome produced from pBS-PyI-VP1_only plasmid. We decided to test that these LoxP sites did not alter the production of viral mutant. Both variants of genome production were further compared with the novel and standard method for production of wild-type virus genome. The genomes after re-ligation (designated as ligation mixtures) from plasmid backbones are shown in Figure 5.5. Transfections are summarized in Tab 5.1.

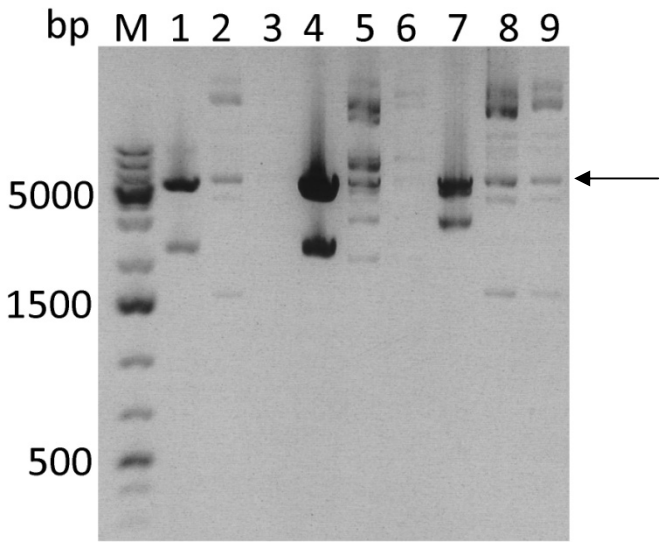


Figure 5.5: Agarose gel electrophoresis with ligation mixes of pMJG, pMJ-VP1, pBS-PyI-VP1_only. All plasmids were digested with restriction enzymes EcoRI or SalI and XhoI for pMJG/pMJ-VP1 and pBS-PyI/pBS-PyI-VP1_only, respectively. Digested plasmids were purified and ligated in large volume to prevent formation of concatemers or religation with plasmid backbone. 1 - pMJG cleaved with EcoRI; 2 - 1 µl of pMJG ligation mix; 3 - 0.1 µl of pMJG ligation mix; 4 - pMJ-VP1 cleaved with EcoRI; 5 - 1 µl of pMJ-VP1 ligation mix; 6 - 0.1 µl of pMJ-VP1 ligation mix; 7 - pBS-PyI-VP1_only cleaved with SalI and XhoI; 8 - 1 µl of pBS-PyI-VP1_only ligation mix; 9 - 0.1 µl of pBS-PyI-VP1_only ligation mix. Bands are representing mouse polyomavirus genome (black arrow - 5,307 kbp long), vector plasmid, concatemers or religated vector plasmid with genome.

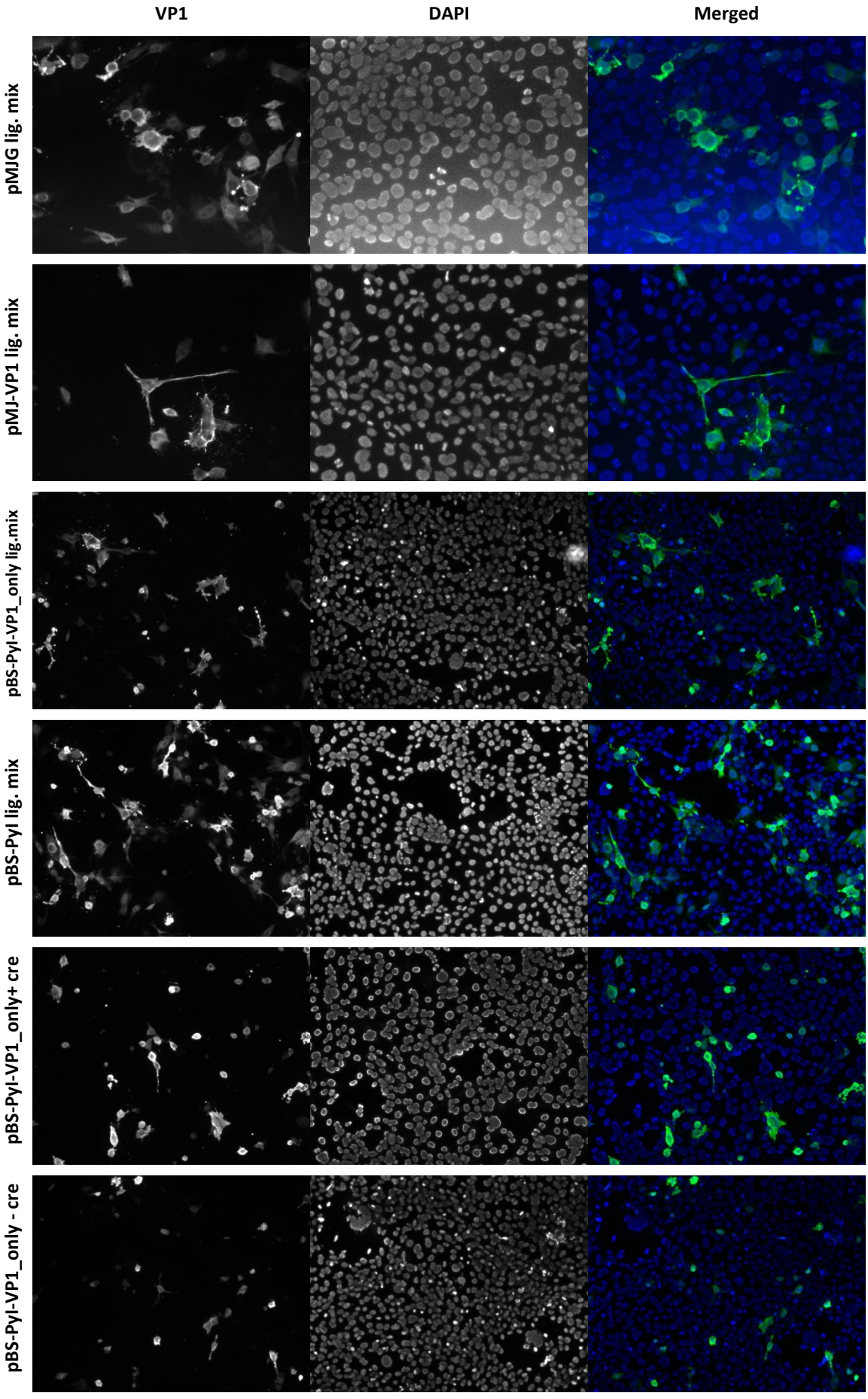
	Transfected DNA	Description
1.	pMJ-VP1 lig. mix (3 µl ~ 6 µg)	Genome prepared from original pMJ-VP1 plasmid by excision and re-ligation method
2.	pBS-PyI-VP1_only lig. mix (3 µl ~ 6 µg)	Genome prepared from pBS-PyI-VP1_only plasmid by excision and re-ligation method
3.	pBS-PyI-VP1_only plasmid (3 µg)+ pPuro.Cre empty plasmid (3 µg)	pBS-PyI-VP1_only plasmid co-transfected with Cre-expressing plasmid pPuro.Cre empty
4.	pBS-PyI-VP1_only plasmid (3 µg)+ pGL3 control plasmid (3 µg)	pBS-PyI-VP1_only plasmid co-transfected with "mock DNA (pGL3 control plasmid)"
5.	pCALNL-dsRed plasmid (3 µg)+ pPuro.Cre empty plasmid (3 µg)	pCALNL-dsRed plasmid express dsRed fluorescent protein after *in vivo* recombination due to the expression of Cre recombinase from pPuro.Cre empty plasmid
6.	Mock	Cells transfected without added DNA
7.	pMJG ligation mix (3 µl ~ 6 µg)	Genome prepared from original pMJG plasmid by excision and relegation method
8.	pBS-PyI ligation mix (3 µl ~ 6 µg)	Genome prepared from pBS-PyI plasmid by excision and re-ligation method
9.	pBS-PyI plasmid (3 µg) + pPuro.Cre empty plasmid (3 µg)	pBS-PyI plasmid co-transfected with Cre-expressing plasmid pPuro.Cre empty
10.	pBS-PyI plasmid (3 µg) + pGL3 control plasmid (3 µg	pBS-PyI-VP1_only plasmid co-transfected with "mock DNA (pGL3 control plasmid)"
11.	pMaxGFP (Lonza) (6 µg)	GFP transfection serves as control of efficiency of transfection

Table 5.1: List of transfections of 3T6 cell. Transfection were performed as described in chapter 4.3.5.4 with 6 µg per 10^6 cells. Mock transfection serves as control. Transfection with pCALNL-dsRed with pPuro.Cre empty serves as control to co-transfetions and as a control to Cre recombinase production. Plasmid pGL3 control serves as mock DNA to co-transfections. Virus production from pBS-PyI and pBS-PyI-VP1_only should be enabled only in cells co-transfected with pPuro.Cre empty plasmid.

Plasmids with wild-type MPyV pBS-PyI and plasmid with mutated MPyV pBS-PyI-VP1_only were transfected into 3T6 cell via Amaxa nucleofection system. Plasmids were co-transfected with pPuro.Cre empty plasmid which expresses Cre recombinase in cells, or with pGL3 plasmid which serves as stuffer DNA instead of pPuro.Cre empty. Transfected cells were seeded on cover slips for subsequent immunofluorescence analysis (Fig. 5.6) as well as on petri dishes for biochemical analysis of virus protein production (Fig. 5.7).

Transfected cells on petri dishes were harvested 48 hours post transfection. Sample of protein lysates from each transfection were loaded on SDS-PAGE and western blot was performed with antibody against VP1 (αPyVP1-A) and VP2/3 (αPyVP2/3-2C8). Western-blot (Fig. 5.7) shows that VP2 and VP3 were expressed only in cells transfected with wild-type genomes (pMJG ligation mixture and pBS-PyI ligation mixture, see table 1 for description). Purified wild-type virus was added as positive control for antibodies.

Cells seeded on cover-slips were cultivated for 36 hours and after incubation, indirect immunofluorescence was performed to detect VP1 (αPyVP1-D4). Indirect immunofluorescence shows that VP1 is produced in all transfections with all plasmids or ligation mixtures (except pCALNL-dsRed and pMaxGFP). Transfection was carried on 3T6 cells which do not express Cre recombinase. For production of virus co-transfection with plasmid expressing Cre recombinase (p.Puro.Cre empty) is essential. Low level of VP1 expression is observed in transfection with plasmid pBS-PyI or pBS-PyI-VP1_only without pPuro.Cre empty co-transfection due to the plasmid design (Hron, 2013).


VP1
DAPI
Merged
pMJG lig. mix
pMJ-VP1 lig. mix
pBS-Pyl-VP1_only lig.mix
pBS-Pyl lig. mix
pBS-Pyl-VP1_only+ cre
pBS-Pyl-VP1_only - cre

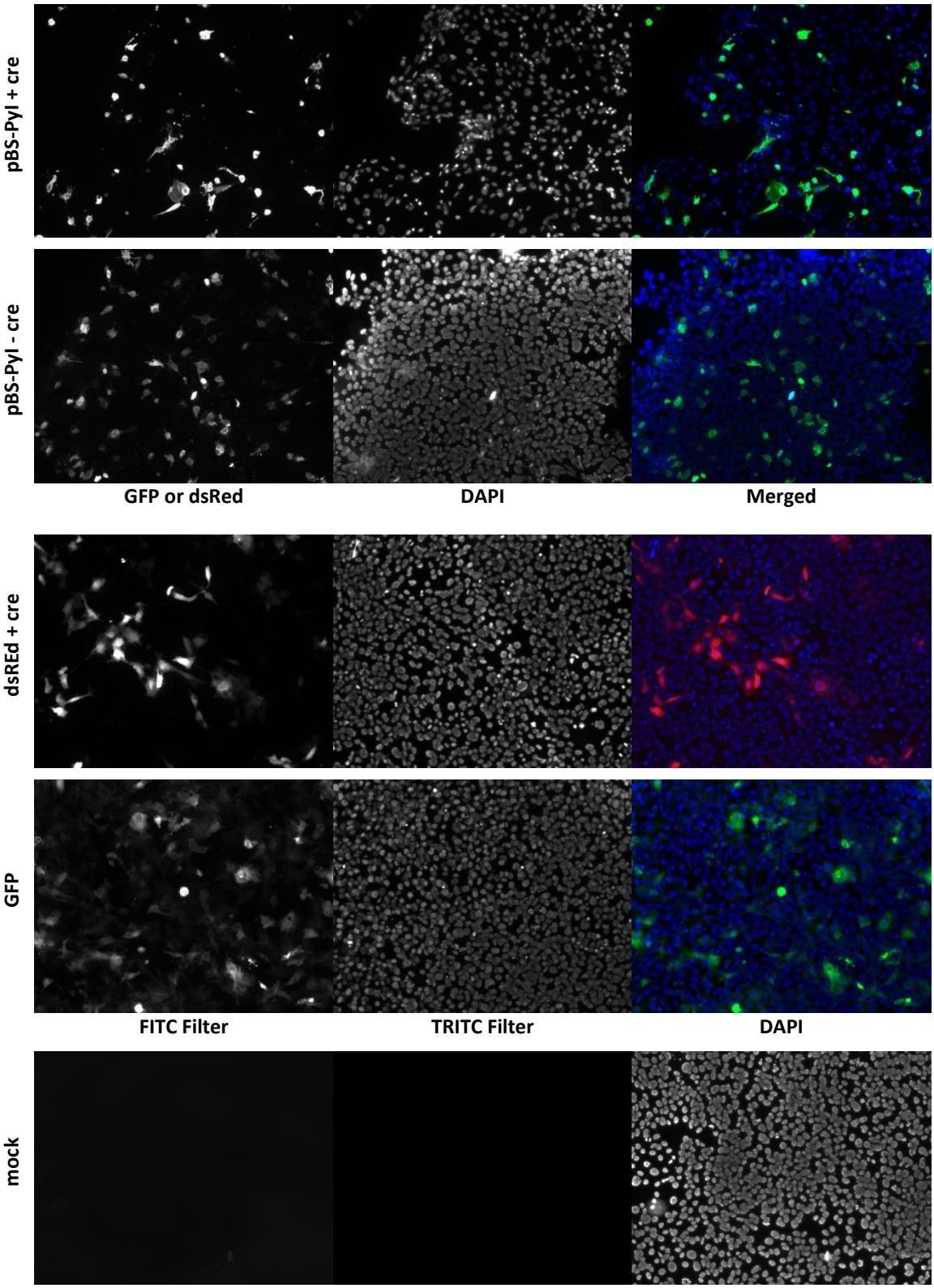


Figure 5.6: Indirect immunofluorescence of transfected cells after 36 hours. Production of VP1 protein is present after 36 hours post transfection in cells. Production should not be present in cells which were not co-transfected with pPuro.Cre empty plasmid. Without Cre recombinase VP1 is produced in pBS-PyI construct but the fluorescence signal is lower and less frequent.

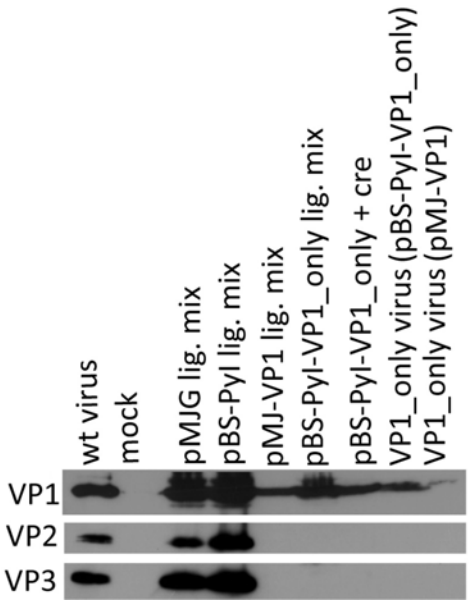


Figure 5.7: Western blot with selected samples of lysates from cells transfected with plasmids listed in Tab 5.1 and isolated virus as controls. Purified wild-type virus serves as positive control for capsid proteins. Subsequently VP1_only mutant viruses were produced with both systems (pBS-PyI-VP1_only and pMJ-VP1) (chapter 5.1.4) and added as samples to western-blot to confirm that there is no VP2 or VP3.

The rest of cells harvested from dishes were lysed by freeze-thaw cycle and used for re-infection of cells to compare novel and standard wild-type virus production systems and verify the fact that VP1_only virus does not initiate infection. Cells were fixes 24 hours post infection and labelled with antibody against LT antigen (rat αLT1). Cells transfected with plasmids pBS-PyI and pBS-PyI-VP1_only without pPuro.Cre empty shows no level of infection and confirms that there is only leaky production of VP1 not the whole virus We have confirmed that there is no contamination with wild-type virus in transfected cells. Secondly, we have compared production of infectious progeny from pMJG ligation mixture, pBS-PyI ligation mixture and pBS-PyI co-transfection with pPuro.Cre empty production systems (Fig 5.8).

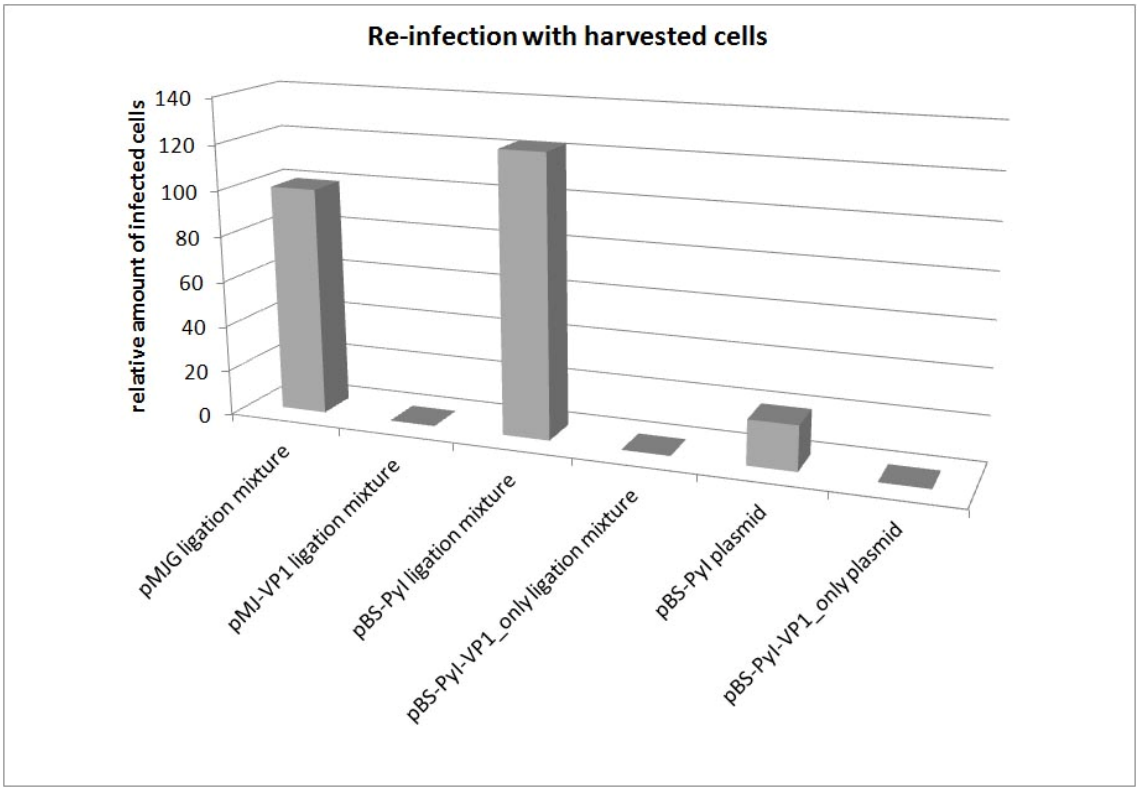


Figure 5.8: Relative number of cells infected (y axis) by 0.05 ml of lysate of transfected cells (x axis). Indirect immunofluorescence was performed with antibody against LT antigen. Minimum 600 cells were counted. pBS-PyI plasmid and pBS-PyI-VP1_only plasmid column stand for re-infection from co-transfected cells with pBS-PyI plasmid/PyI-VP1_only plasmid and pPuro.Cre empty. Number of infected cells is related to the transfection efficiency of harvested cells. Values were normalized to pMJG ligation mixture (wild-type virus produced with standard method).

5.1.3. Characterization of Cre recombinase production cell line for mutant virus production.

We tested several cell lines producing Cre recombinase for our mutant virus production. These cell lines were previously prepared in our lab (Hron, 2013). 3T6 cell line was transfected with expression plasmid for Cre recombinase and production cell line was cultivated in selection media. Expression of Cre recombinase was constitutive (transfection with pPuro.Cre empty) or inducible with Tet-Off promoter (transfection with pNit-Cre). Due to the lack of reliable antibody against Cre recombinase, production of Cre recombinase was tested with transfection with dsRed plasmid. If Cre recombinase is produced in the cell, the dsRed plasmid is recombined and fluorescent protein is produced. We also tested the transfection efficiency with plasmid pMaxGFP. The production of virus was tested with transfection of viral genome (produced from pMJG or pBS-PyI) and co-transfection of pBS-PyI plasmid with pPuro.Cre empty or pGL3 control.

We analyzed 3T6 transfected with pPuro.Cre empty (designed as P1 and P2 cells). Furthermore we tested 3T6 cell line transfected with pNit-Cre designed as T2. These cell lines were transfected via nucleofection (Amaxa) as described in chapter 4.3.5.4. First, cells were transfected with dsRed plasmid and pMaxGFP to determine Cre recombinase production and transfection efficiency, respectively. Next, we transfected cells with viral genomes or with pBS-PyI plasmid. Transfected cells were harvested 7 days post transfection and lysed by 3 freeze-thaw cycles.

To determine efficiency of virus production, 3T6 cells were re-infected with cell lysate of harvested cells (50 µl). Cells were fixed 48 hours post infection and indirect immunofluorescence was performed with antibody against VP1 (αVP1 D4). The results are shown in Figure 5.9. Transfection with viral genome prepared from pMJG provides three times more progeny than transfection with viral genome prepared from pBS-PyI in T2 cell line. Co-transfection of pBS-PyI with pPuro.Cre empty provides more infectious progeny that transfection with viral genome prepared from pBS-PyI and 5 times more infectious progeny than co-transfection with control plasmid. Transfection of pBS-PyI without control plasmid provides slightly more infectious progeny. P1 and P2 cells provide more infectious progeny than T2 cells but the extremely slow growth of P1 and P2 cells makes them unusable for virus production.

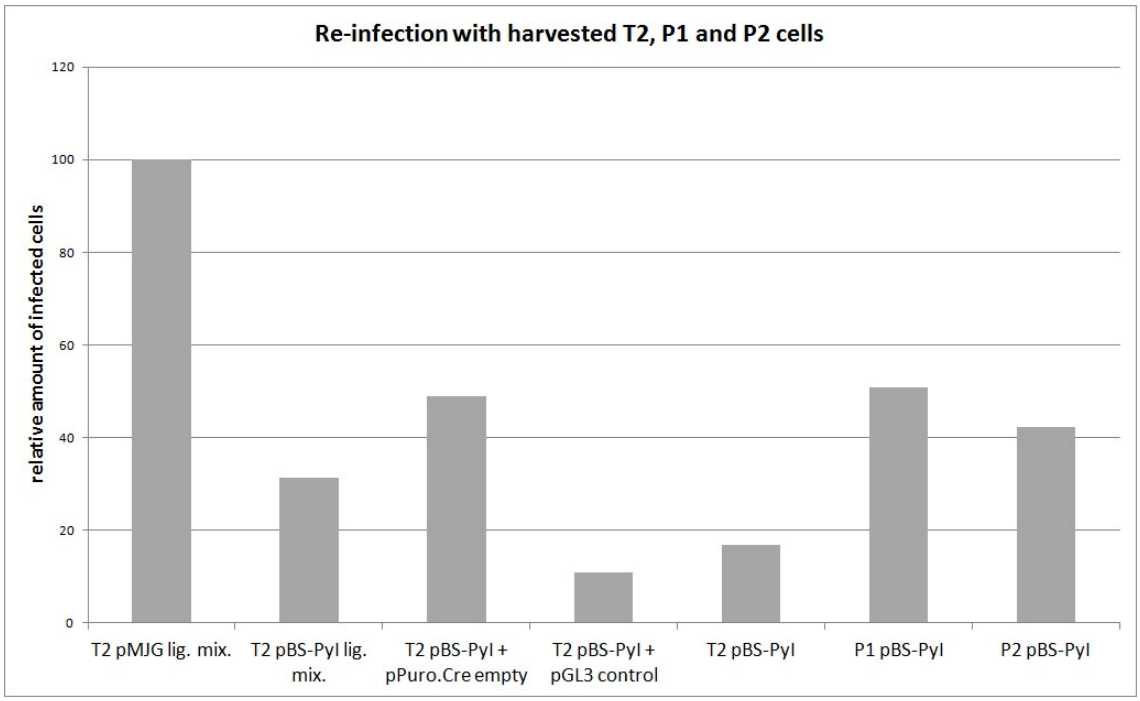
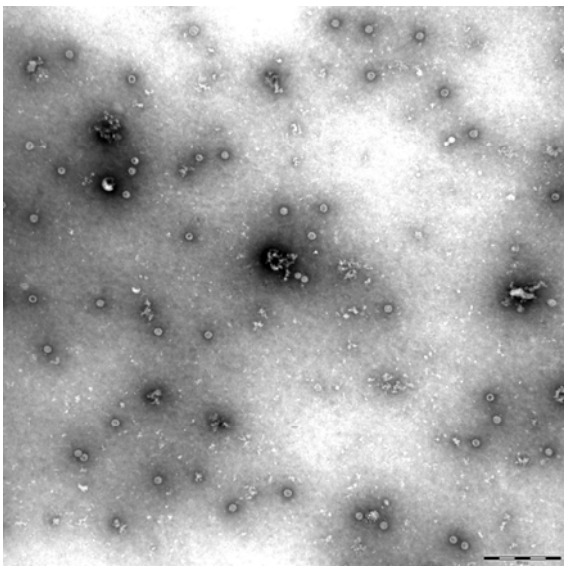


Figure 5.9: Relative amount of infected 3T6 cells in samples. Cells on cover slips in 24-well plate were infected with 50 µl of harvested cells from transfection. Description in x axis means transfected cell line and transfected DNA. Cells were fixed 48 h.p.i. and labelled with antibody against VP1 (αVP1 D4). Minimum 700 cells were counted for each sample. Amount of infected cells are related to the transfection efficiency in harvested cells. Values were normalized to pMJG ligation mixture.

5.1.4. Characterization of VP1_only virus

We decided to compare efficiency of mutant virus production between novel Cre-LoxP system and "standard" procedure. This means that the genome was either prepared by excision and relegation method from original pMJ-VP1 vector and transfected in 3T6 cells or the pBS-PyI-VP1_only vector was directly transfected into Cre-expressing T2 cells. The same amount of cells was used in both experiments and cells were harvested 3 or 4 days post transfection, respectively. As previously found (Hron, 2013) the Cre-LoxP system requires longer cultivation for efficient virus production. Mutant virus was isolated according the standard protocol (see chapter 4.3.4.3) by cesium chloride gradient centrifugation. Gradients were separated into a number of fractions and both viral preparation were collected in fraction according the cesium chloride density – the first fraction designated as "full" virions (refractive index between 1.364 - 1.367) and the second fraction which should contain "empty capsids" (refractive index between 1.360 - 1.363). We have determined number of particles with hemagglutination assay for each preparation. Full fraction of VP1_only virus produced with pMJ-VP1 lig. mix contained 8×10^9 particles/ml and empty fraction contained 7.2×10^{10} particles/ml. Full fraction of VP1_only virus produced from pBS-PyI-VP1_only plasmid contained 6.4×10^{10} particles/ml and empty fraction contained 8×10^9 particles/ml. These data show that VP1_only virus yield was low with both methods of production. Furthermore the electron microscopy revealed that both preparations contain large amount of disassembled material (Fig. 5.10). This suggests that virions are unstable.

VP1_only virus (from pMJ-VP1 system)



VP1_only virus (Cre-LoxP system)

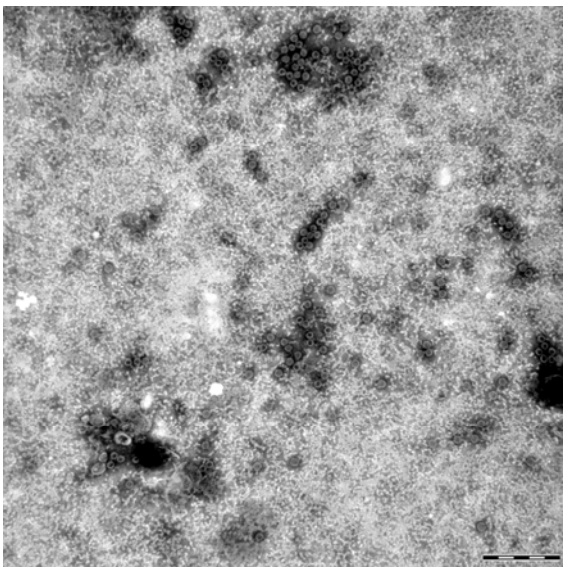


Figure 5.10: Electron micrograph of VP1_only virus from pMJ-VP1 and Cre-LoxP production system. Protein concentration of inoculum produced with pMJ-VP1 system is 45 µg/ml and protein concentration of inoculum produced with Cre-LoxP system is 236 µg/ml. Both pictures and concentration are from fraction in which we expected full particles. Magnification 40 000x. Bar 500 nm.

Next, we wanted to determine whether the virions contain viral genome or host cell DNA. For this purpose we isolated DNA from VP1_only virus (generated from pMJ-VP1 ligation mixure) subjected isolated DNA to hybridization with probe specific for viral genome. The virus preparation was treated with DNase I prior to DNA isolation to remove random DNA which can be associated with capsids. We have performed hybridization with DIG labelled probe (fragments of plasmid pMJG). DNA was denaturated in pre-hybridization step which is part of the manufacturer´s protocol for detection. As positive control, we used DNA isolated from wild-type virus. As negative control, we used irrelevant DNA, plasmid pFastBac Dual. We have dotted an equal amount of DNA for each sample on nylon membrane. The results (Fig. 5.11) confirm that there is a viral DNA packaged in mutant virions but signal was weaker compared to wild-type virus. This suggests that virions may be packed with the random DNA.

For quantification of viral genome in capsids we tried to utilize qPCR with primers MPyV-OriDetection-Fw and MPyV-OriDetection-Rw that amplify Ori region of MPyV. For this, we isolated DNA from DNAse I treated virions. In contrast to wild-type virions we repeatedly obtained very low amount of DNA from mutant virions. Furthermore, the results of qPCR (Fig. 5.12) showed great variation between independently repeated experiments. This suggests that genome in mutant virions is more accessible to DNase I and qPCR does not provide a reliable data. These data, however, indicated that around 75% of viral DNA is packed into capsids produced with pMJ-VP1 system and only 47% of viral DNA is in the VP1_only virus produced with Cre-LoxP system (Fig. 5.12). Hybridization indicate that there is should be more viral DNA in capsids. We have detected the number of the Ori sequences but we detected whole genome with hybridization.

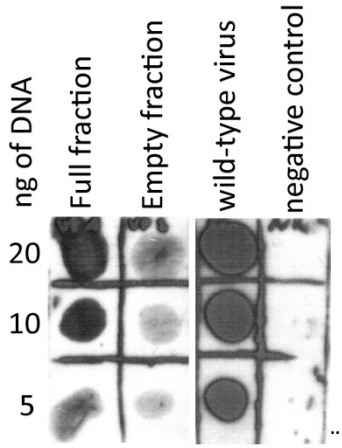


Figure 5.11: Hybridization of isolated DNA with digoxigenin labelled fragments of pMJG. As negative control was used pFastBac Dual. Signal for DNA isolated from VP1_only virus (pMJ-VP1 system) is lower than in wild-type virus.

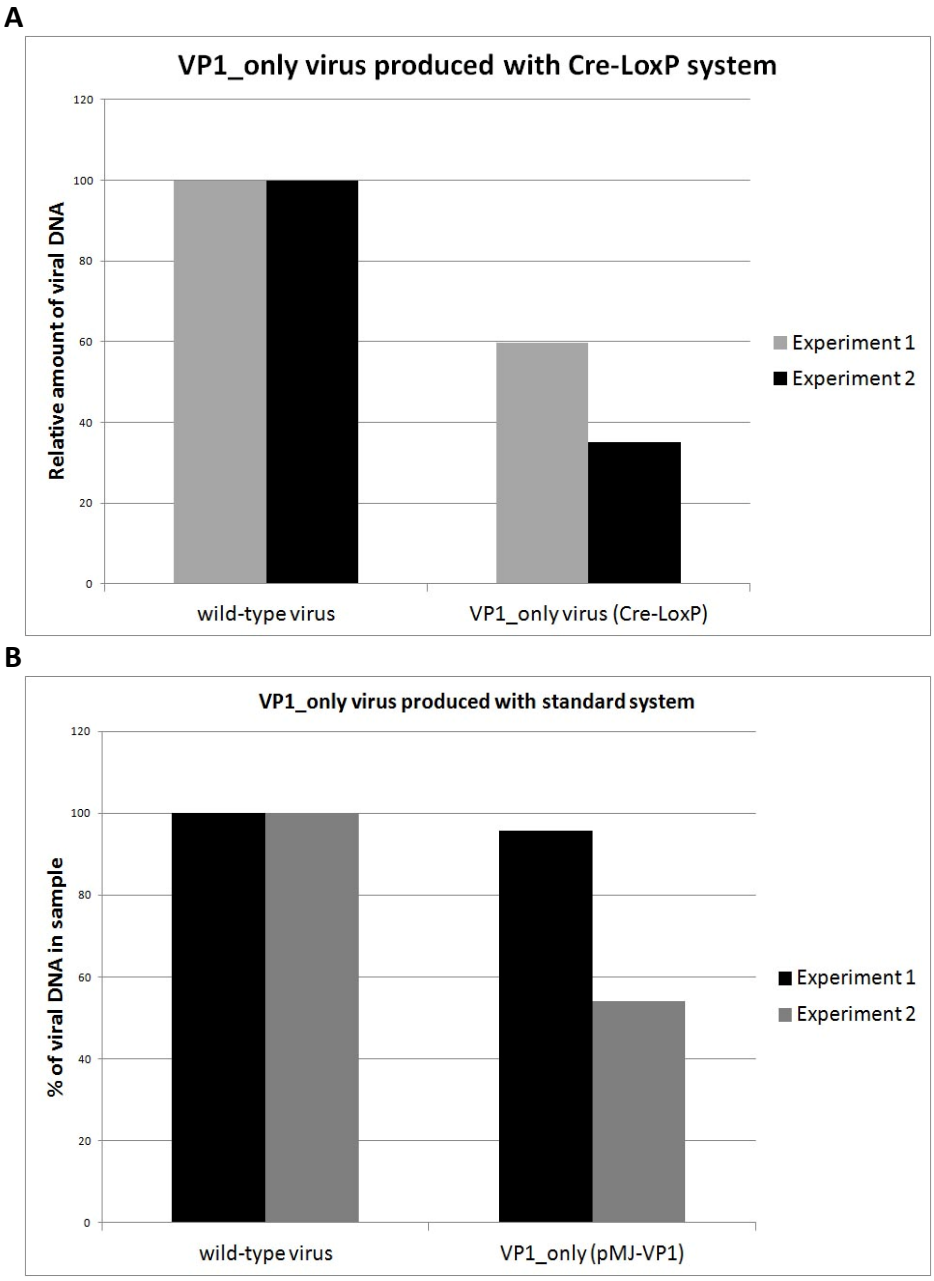


Figure 5.12: Normalized concentration of the viral DNA related to input DNA. Concentration of viral DNA was calculated from qPCR data and normalized to input DNA concentration to determine ratio between viral DNA and random DNA. These values were further normalized to DNA amounts obtained from wild type virus sample (represented by value 100% on the y-axis in the graph) to determine the relative proportion viral genome in mutant virions. **A:** Cre-LoxP system of production. **B:** Standard system of production.

We measured the concentration of protein in viral isolation for wild-type virus, VP1_only virus produced from pMJ-VP1 system and VP1_only virus produced from Cre-LoxP system. Also we measured concentration of DNA isolated from these viruses. These data with

the data we collected from qPCR and hemagglutination assay provide us with information how many capsids are in virus preparation per one genome (Tab. 5.2).

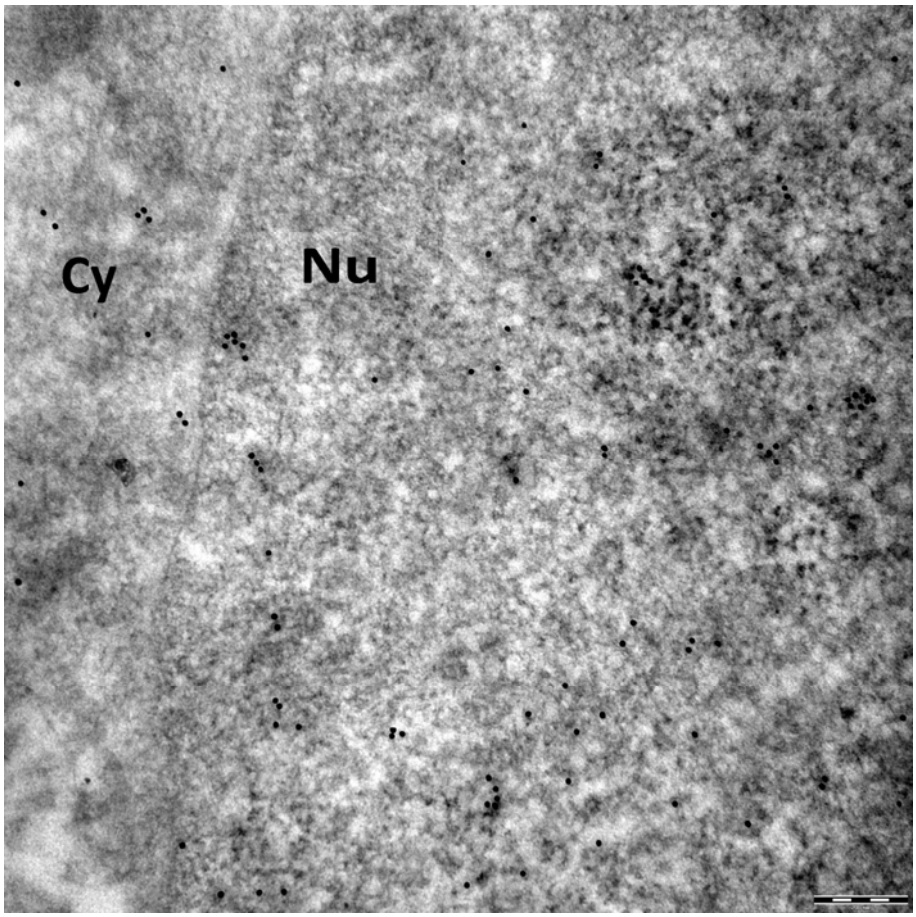
	Wild-type virus	VP1_only (pMJ-VP1)	VP1_only (Cre-LoxP)
ng of protein needed for 1 ng of DNA	48,030	984,380	31375,000
ng of protein needed for 1 ng of viral DNA	48,030	1316,368	66191,983
number of particles in 1 ng of proteins	43697478,992	177777,778	271186,441
number of particles in 1 ng of proteins for 1 ng of viral DNA	2098789915,966	234020980,060	17950368304,370
number of particles for 1 genome	11,428	1,274	97,739

Table 5.2: Table indicatng number of capsids per 1 viral genome. We have calculated the values with presumption that 100% of DNA isolated from wild-type virus is of viral origin. For calculations are used data from qPCR and hemagglutination assays.

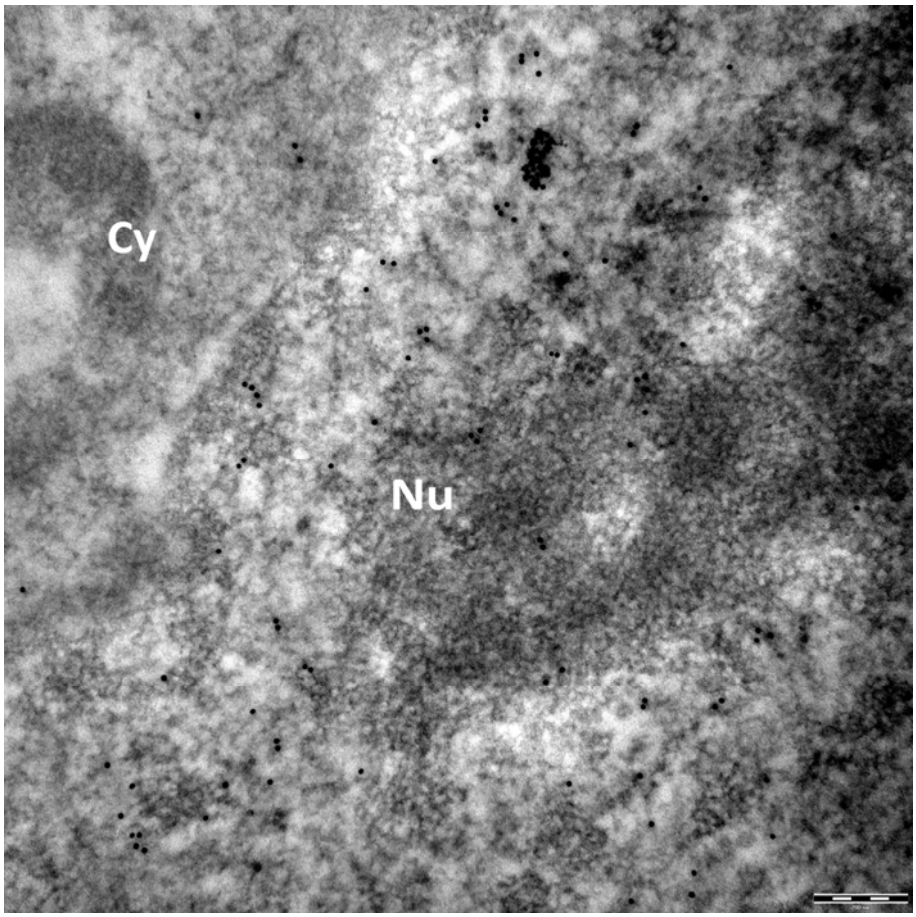
5.1. 5. Electron Microscopy of cells transfected with pMJ-VP1

With qPCR we have verified that VP1_only virus contain a small portion of viral DNA. Based on the data from qPCR we assumed that VP1 protein must be translocated to the nucleus. But unpublished data of Lenka Horníková suggest that VP1 alone cannot translocate to the nucleus. For translocation, VP1 needs at least one minor protein. To verify presence of VP1, we transfected cells via Amaxa nucleofection system with viral genomes produced from pMJG (wild-type virus) and pMJ-VP1 (VP1_only virus) plasmids. Transfected cells were seeded on petri dish. 48 h.p.i. were cells fixed and embedded in resin. First, we applied embedding into Agar 100 resin (Epon resin) to preserve morphology as much as possible but viral particles were hard to distinguish in the fixed cells and we were not sure if the structure we saw was virus. To clearly distinguish viruses, we needed to detect VP1 on sections but glutaraldehyde fixation (see chapter 4.3.10.1) which is used in Agar 100 embedding does not allow detection with antibody due to the destroyed epitopes for antibodies. We decided to transfect cell again but this time we embedded the cells in LR white resin and performed immunogold labelling (αVP1 rabbit polyclonal IgG and αrabbit IgG gold conjugate (10 nm)). We encountered the same problem as in the Agar 100 embedding. We cannot distinguish the viral particles by eye but we have focused on the abundance of antibody against VP1. Compared to negative control (mock transfection), the abundance of VP1 antibody in the nucleus was much higher in both samples (Fig. 5.13).

pMJG ligation mixture transfection



pMJ-VP1 ligation mixture transfection



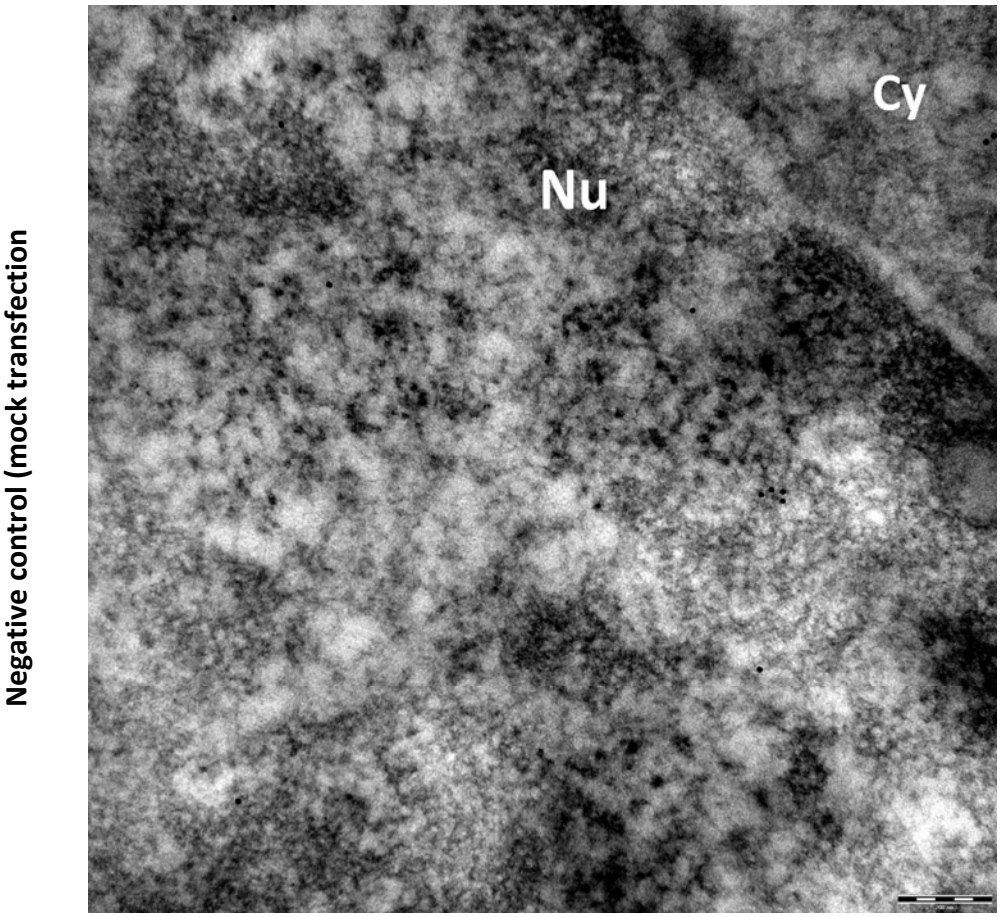


Figure 13: Electron micrographs from sections of cells transfected with pMJG or pMJ-VP1 ligation mixtures. Cells were fixed 48 h.p.i. and labelled with antibodies αVP1 rabbit IgG polyclonal and αrabbit IgG gold conjugate (10 nm). Cy- cytoplasm, Nu - nucleus. Magnification 75 000x. Bar 200 nm.

5.2 Production of polyomavirus with genome modified by thymidine analogues

Virus trafficking to the nucleus has been thoroughly studied in our lab and we have decided to prepare a polyomavirus with incorporated thymidine analogues in its genome for this purpose. We used 5-Bromo-2'-deoxyuridine (BrdU) or 5-Ethynyl-2'-deoxyuridine (EdU) for our experiments. These analogues can be detected with antibody against BrdU (detects BrdU as well as EdU) or via click chemistry (EdU). This method will help us to track the virus in the disassociated form.

5.2.1 Virus production and characterization

EdU and BrdU are metabolized *in vivo* by cellular enzymes and incorporated in host DNA as well as in viral genome in infected cells by cellular polymerases. Cells were infected with high multiplicity of infection (MOI). Usually, cells are infected with MOI about 0.01 for 10 days (until the death of cells) but production of so called “BrdU virus” has to be modified due to the mutagenicity of BrdU. To produce BrdU virus, we infected cells with MOI 5 as described in Kuksin and Norkin (2012). Because we wanted to compare BrdU virus and virus with EdU incorporated in its genome (designated as “Edu virus”). EdU virus was produced in cells infected with same the MOI as BrdU virus. We have incubated infected cells for 5 days in the dark (until the cell death) and EdU/BrdU was added two times during incubation (see chapter 4.3.4.1). Viruses were isolated (Fig. 5.14) from cells and media with minimum exposure to light EdU/BrdU are light sensitive according the standard protocol (see chapter 4.3.4.3). The removal of cellular DNA is not a part of standard purification protocol however; this DNA could cause false positivity in our experiments because it probably contains EdU/BrdU. To remove random DNA we treated virions with DNase I which was subsequently removed by centrifugation through sucrose cushion (Fig. 5.14).

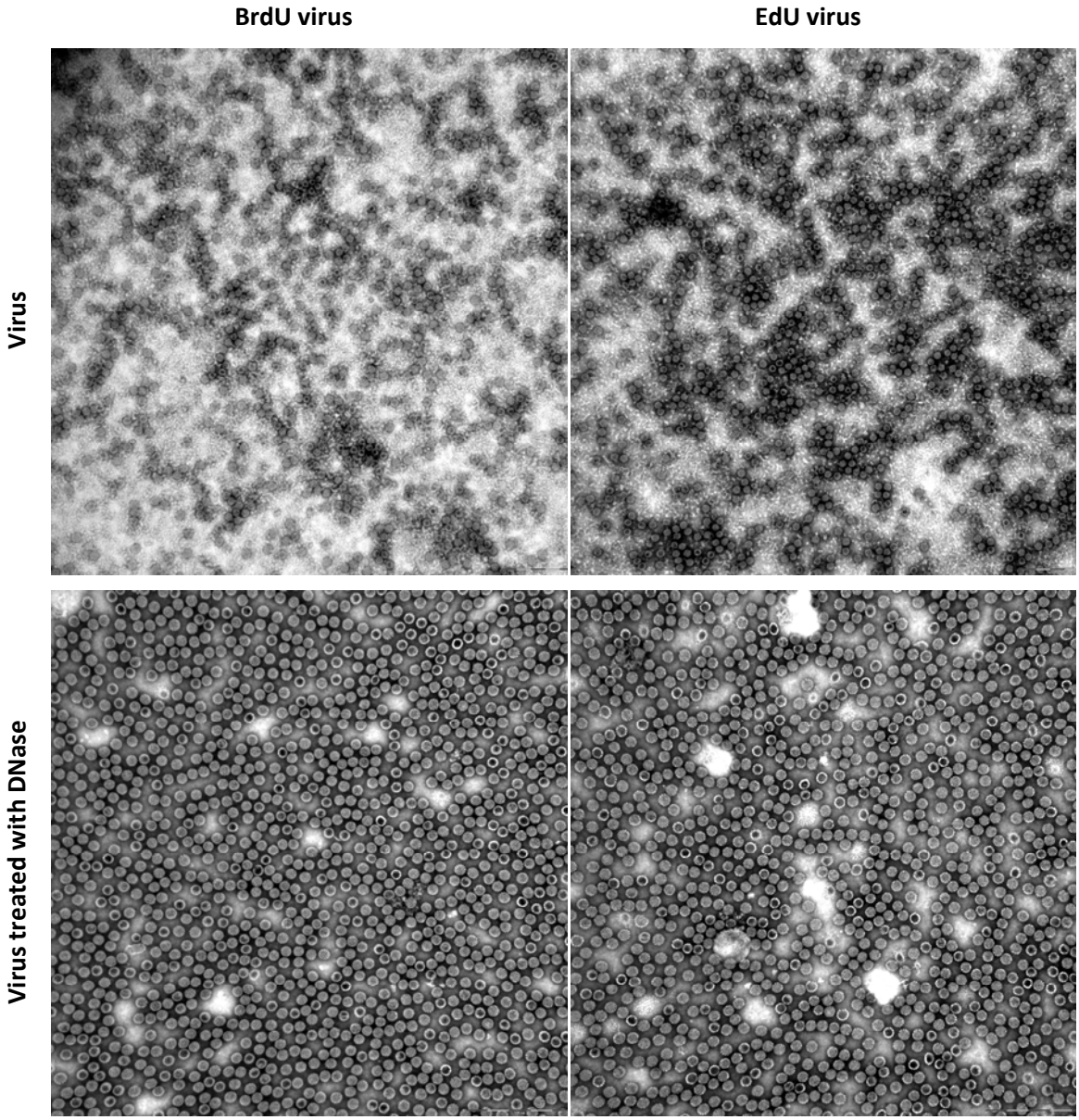


Figure 5.14: Negative staining of BrdU/EdU virus and BrdU/EdU DNased virus. BrdU/EdU virus is showed with magnification 30,000x and BrdU/EdU DNased virus is showed with magnification 60,000 x.

Further, we wanted to determine if DNA in virions contains EdU or BrdU. In the theory, incorporated thymidine analogues could alter the packing of viral DNA into capsid. EdU/BrdU could inhibit binding of VP1 protein to DNA which would mean that random DNA without analogues is packed in capsid. Also, EdU/BrdU could abolish binding of viral DNA to the capsids.

Therefore, we isolated DNA from virions. Samples of DNA isolated from BrdU virus, EdU virus and wild-type virus (i.e. without thymidine analogue) were hybridized with DIG labelled probes prepared from fragments of pMJG. The hybridization assay (Fig. 5.15 A) confirmed that the DNA is of virus origin and in the same quantity as wild-type virus. As

negative control we used irrelevant DNA (plasmid pFastBac Dual). To test the presence of BrdU or EdU in virions we performed immunodot-blot assay with isolated DNA and antibody against BrdU (Abcam Ab 6326) which cross-react with EdU. Dot-blot proves that in the BrdU/EdU virus contains appropriate analogues. As positive control was used DNA isolated from cell treated with BrdU (the presence of BrdU in cells was previously confirmed with immunofluorescence assay (data not shown)). As negative control we used plasmid pMaxGFP (Lonza) (Fig. 5.15 B).

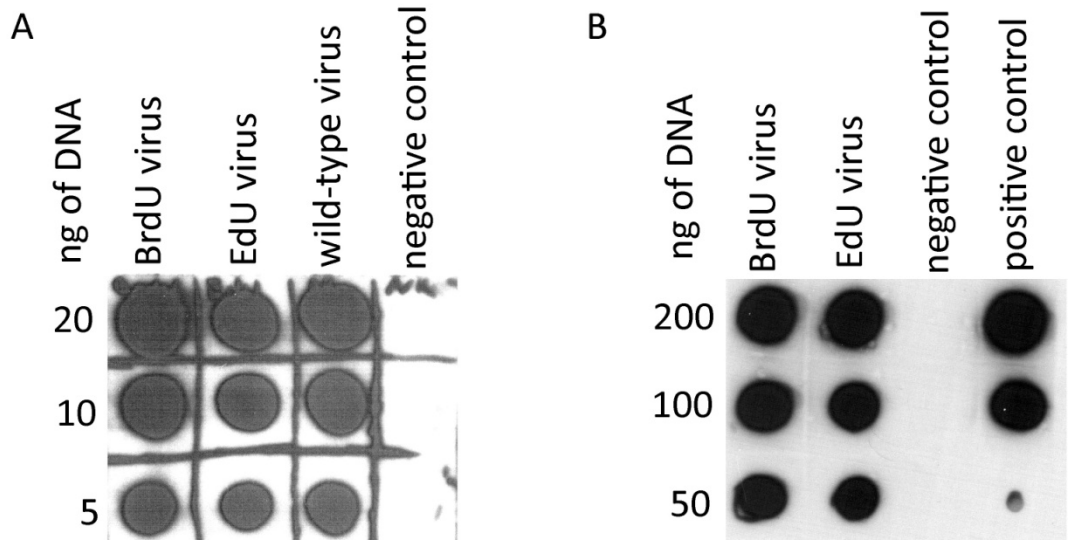


Figure 5.15: Characterization of DNA isolated from BrdU and EdU virus. A: Hybridization with polyomavirus DIG-labelled DNA probe (pMJG labelled with digoxigenin). As negative control we used pFastBac Dual. B: Immunodot-blot assay detected with antibody αBrdU. As negative control we used pMaxGFP (Lonza) and as positive control we used cellular DNA with BrdU analogue. From other experiments we know that 50 ng of cellular DNA is the detection limit. The difference between viral DNA and cellular is probably the frequency of BrdU incorporation.

Next, we determined viral titre and hemagglutination titre before and after the DNase I treatment. After treatment with DNase I, virus was ultracentrifuged 2 times through sucrose cushion. When we calculated ratio between infectious titre and number of particles before and after DNase I treatment we found out that the infectivity of virus dropped by 70% and 85%, respectively (Tab. 5.3).

Virus	Viral Titre (fu/ml)	Number of Particles per ml	Number of Particles per 1 fu
EdU virus	5.1×10^8	1.15×10^{13}	22 549
EdU virus DNase treated	$9{,}3 \times 10^7$	6.5×10^{12}	69 862
BrdU virus	9.9×10^8	1.34×10^{13}	13 535
BrdU virus DNase treated	$9{,}5 \times 10^7$	8.5×10^{12}	89473

Table 5.3: Table summarize viral titre and number of particles for EdU/BrdU virus and EdU/BrdU virus DNase treated. Viral titre was determined by immunofluorescence assay and number of particles was determined with hemagglutination assay.

5.2.2 Optimization of EdU/BrdU detection method in cells

We had to establish a method for detection of EdU virus and BrdU virus in our lab. Detection of BrdU requires denaturation of DNA to enable access for the anti-BrdU antibody. This makes immunodetection of other cellular markers especially difficult since the protein epitopes could be damaged by denaturation step. We have tested 3 protocols (see chapter 4.3.7.2) for detection BrdU with second antigen but all 3 protocols were incompatible with our antibodies. We have decided to combine two protocols which gave us best results. Protocol using HCl denaturation was best at detection of second antigen and DNase I treatment protocol was best for BrdU detection. After several optimization procedures we succeeded in the detection of BrdU and second antigen.

Next, we infected cells with BrdU virus (20 000 particles/cell) but we encountered a problem with detection limit for BrdU (Fig. 5.16). We have used indirect immunofluorescence to detect BrdU which means that the signal is amplified but the primary antibody can detect BrdU molecules in certain distance from each other (steric limitation). Second problem was that we do not know the frequency of incorporation.

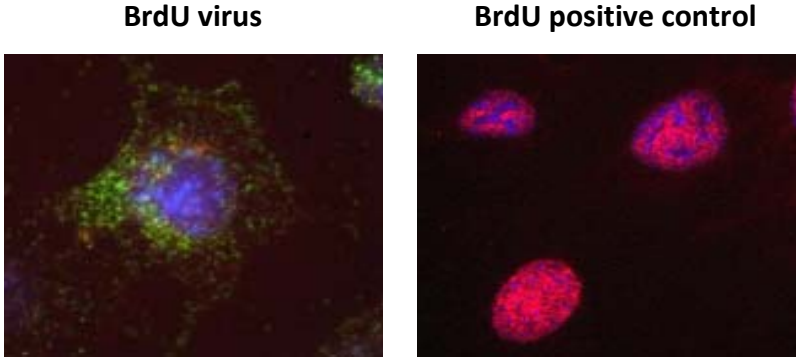


Figure 5.16: Indirect immunofluorescence of cells infected with BrdU virus (20 000 particles/cell). Cells were fixed 8 h.p.i. Positive control was fixes 4 hours post BrdU treatment (1 μg/ml). VP1 was detected with αVP1 D4 (green) antibody and BrdU was detected with Abcam Ab 6326 (red).

For this we prepared the well defined BrdU containg DNA probe. We performed PCR with BrdU triphosphate instead of thymidine triphosphate (TTP). We utilized Deep Vent (exo-) polymerase which should be able to incorporate BrdU. Template for PCR was plasmid RV-1 which has inserted regulatory sequence of MPyV. Primers H20-1PyV-5121Rw and RVprimer_pGL3 are designed to amplify this sequence in RV-1 plasmid. Protocol and PCR mixture (see chapter 4.3.2.7.1) was inspired by (Gierlich et al., 2007; Tabibzadeh et al., 1991). The incorporation of BrdU in PCR product was surprisingly effective (Fig. 5.17).

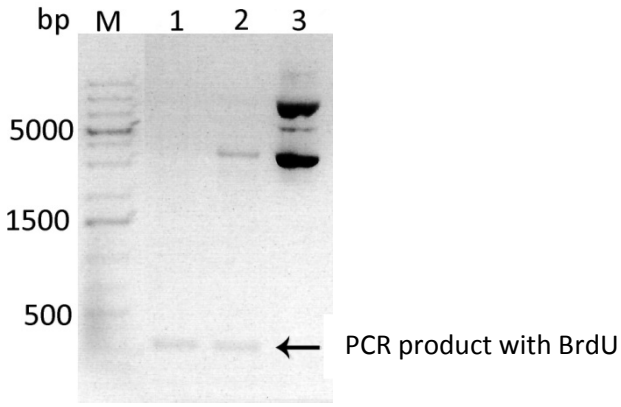


Figure 5.17: Gel with PCR product containing BrdU. Product is 265 bp long and contains 66 BrdU. We have amplified the product from two concentrations of RV-1 template - 50 ng (1) or 400 ng (2). 3 - RV-1 template

The reliable detection of PCR probe was achieved when 500 ng of probe was used per transfection. This probe is approximately 20 times smaller than MPyV genome and therefore 25 ng of viral DNA will be needed for each transfection. 25 ng of viral DNA equals to approximately 4.3×10^9 copies of genome. Each transfection reaction contained 8×10^4 cells. We calculated that we would need to infect one cell with approximately 50 000 genomes to reliably detect BrdU signal. However we also noticed that a lot of DAPI labelled transfected DNA can be seen (Fig. 5.18) in cells transfected with 1 μg of PCR product but not every DNA was labelled with BrdU antibody. In this case 100% of thymidine was replaced with BrdU so every spot should be green in cells but it is not. We suppose that transfection and infection can significantly differ since the manufacturer of Turbofect (Thermo Scientific) states that compound forms compact stable complexes with DNA which could prevent the exposure of BrdU epitope to the antibody. Nevertheless, the experiment proved that we are able to reliably detect incoming exogenous DNA in cytoplasm with almost no background. Small spots of DNA probe could be detected even in transfections with 1 ng of DNA probe (equals to 100 viral genomes per cell). The transfection with viral DNA isolated Hirt method also suggested that *in vivo* BrdU incorporation into viral genome may not be as efficient as PCR.

	Quantity of transfected PCR product	Quantity of transfected carrier DNA
1.	1 µg	0 µg
2.	0.5 µg	0.5 µg
3.	0.2 µg	0.8 µg
4.	0.01 µg	0.99 µg
5.	0.001 µg	1 µg
6.	0 µg	1 µg
7.	1 µg of viral DNA isolated with Hirt	0 µg

Table 5.4: Quantity of transfected DNA in 3T6 cells.

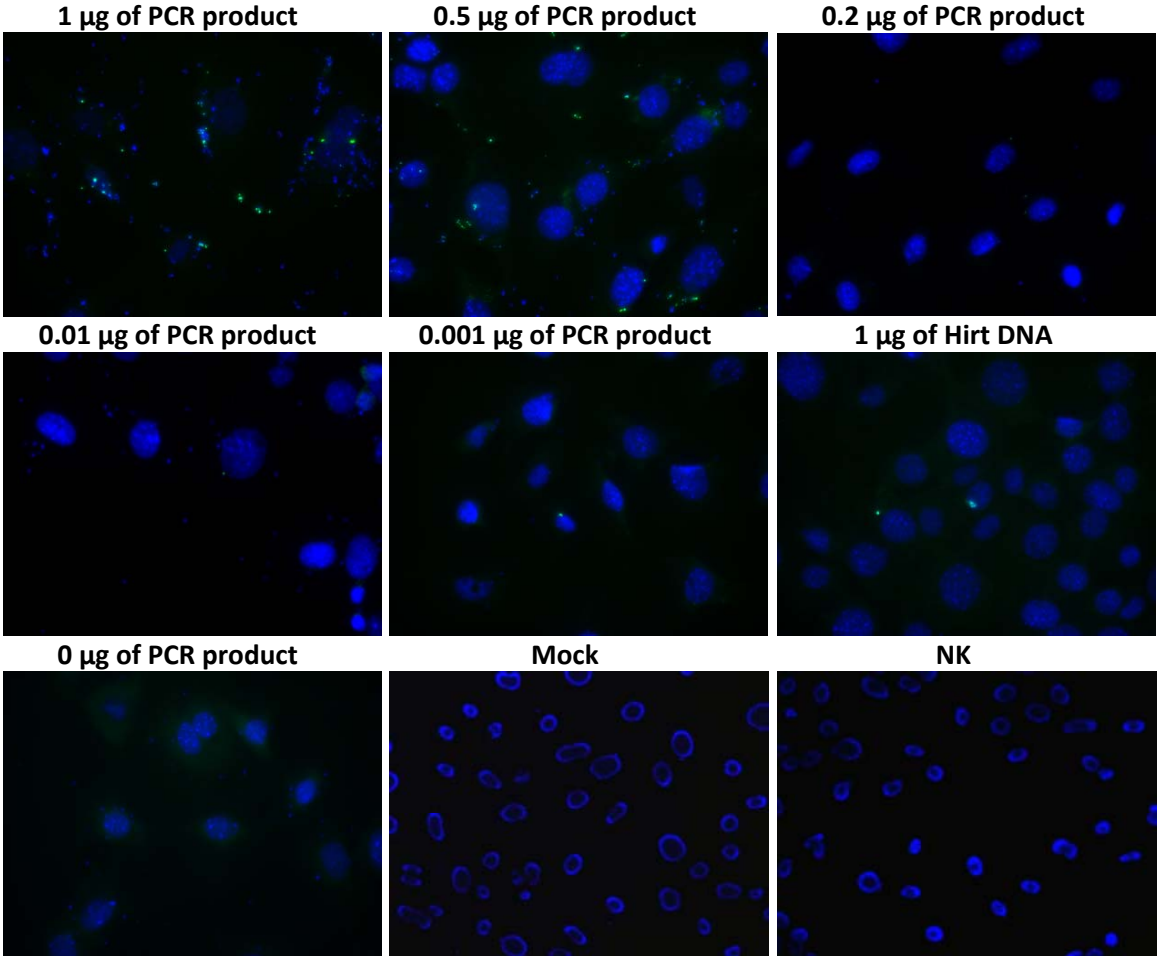


Figure 5.18: 3T6 cells transfected with different amounts (as indicated) of PCR product with incorporated BrdU or with viral DNA with incorporated BrdU (isolated with Hirt method). Green spots are detected BrdU. Blue is DAPI labelled DNA. Blue spots are transfected DNA which is not labelled with antibody.

Detection of EdU has been performed with kit from Molecular Probes for in situ detection of EdU which uses click chemistry. Detection of EdU does not require denaturation of DNA. Alkyne group of EdU is sterically more accessible than BrdU and the dye (Alexa Fluor Azide) is small enough to be clicked in DNA. Each molecule of EdU is clicked with single molecule of Alexa Fluor. But there is the same problem as in BrdU detection. We do not know

the frequency of incorporation in DNA and since the signal is not amplified by antibodies we need lot of molecules of EdU for visible detection. We have performed the same PCR from RV-1 template with EdU analogue but unfortunately the Deep Vent (exo-) polymerase did not incorporate the EdU. We decided to utilize Pwo polymerase to amplify the fragment but with no result. We operated with PCR programme and composition of PCR mixture. Changes in $MgCl_2$ concentration, addition of DMSO or changes in ramps of PCR machine did not help. We decided to add thymidine triphosphate into PCR mixture composition and we found out that added thymidine in concentration 0.02 mM enables the production. We contacted the manufacturer of EdU triphosphate (Jena Bioscience) who confirmed that amplification by PCR only with EdU triphosphate is impossible. We amplified the sequence with mixture of 0.2mM EdU triphosphate and 0.02 mM TTP but we needed to determine number of EdU molecules incorporated in product. PCR product was labelled with Alexa Fluor 594 by click reaction *in vitro* and analyzed by DNA electrophoresis but the results were not convincing (Fig. 5.19). Next, we analyzed clicked DNA in fluorometer to confirm the labelling (Fig 5.20). We wanted to determine number of incorporated EdU molecules in PCR product by measuring base:dye ratio. This ratio can be determined by measuring the absorbance spectrum of DNA at 260 nm and the absorbance of the dye at its absorbance maximum (588 nm). From this data we would calculate the number of EdU in product using formulas according the Beer–Lambert law. Unfortunately, the measurement of samples at dye´s absorbance maximum was repeatedly under detection limit. This suggests that either labelling reaction *in vitro* is not efficient or incorporation of EdU into PCR product was very low. Without these data, we could not determine detection limit with transfection as with PCR product with BrdU incorporation.

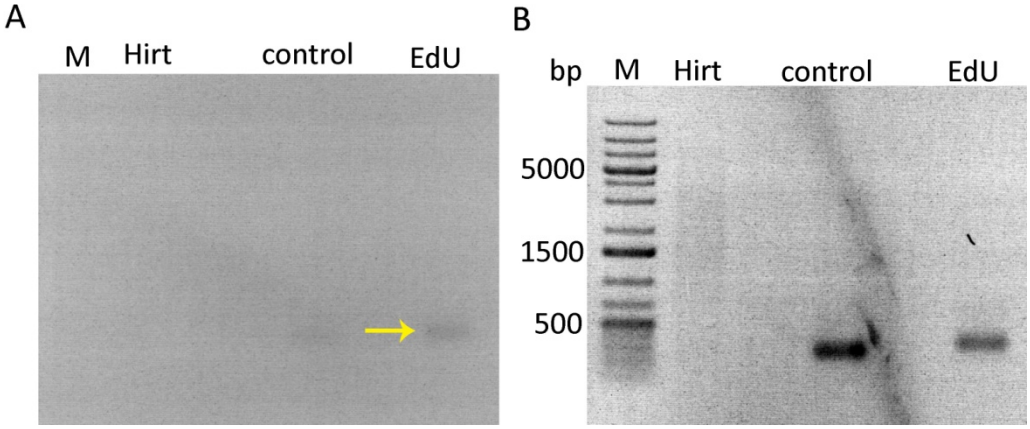


Figure 5.19: Agarose gel electrophoresis of clicked DNA *in vitro.* 3 types of DNA were clicked - viral DNA with incorporated EdU isolated with Hirt method from cell culture (Hirt), PCR product without incorporated EdU (control) and PCR product with incoporated EdU (EdU). **A:** Gel before EtBr staining. DNA which was successfully clicked with Alexa Fluor is visible (yellow arrow). **B:** The same gel stained with EtBr.

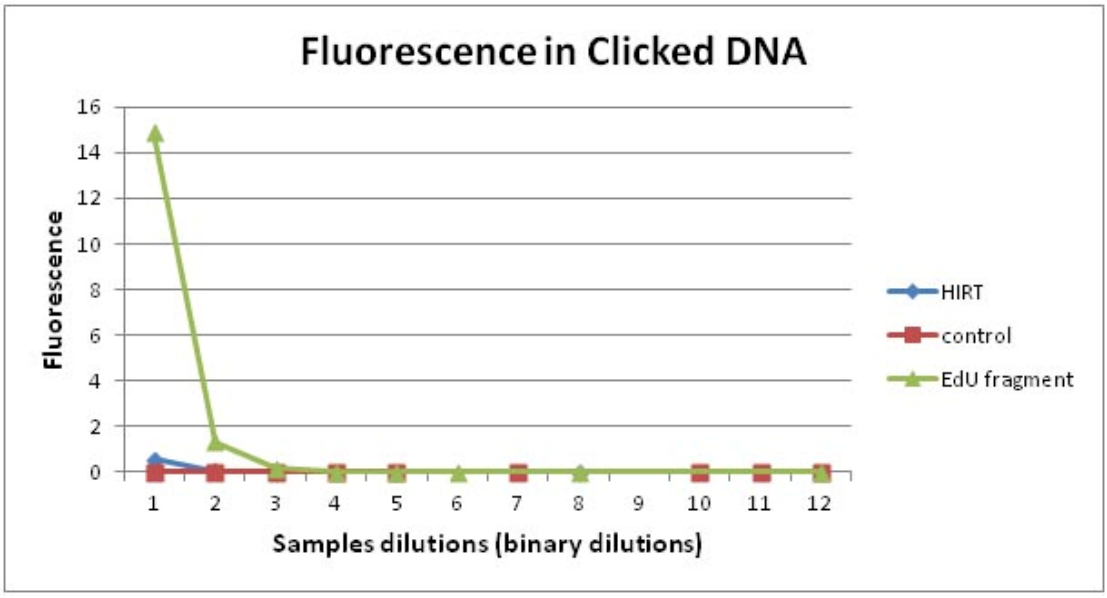


Figure 5.20: Intensity of fluorescence in clicked samples of DNA. Hirt - viral DNA with incorporated EdU isolated with Hirt method from cell culture, control - PCR product without incorporated EdU, EdU - PCR product with incorporated EdU. As control of fluorescence was added diluted Alexa Fluor but the fluorescence was too intense compared to other samples so it is not represented in the graph.

5.2.3 Detection of EdU/BrdU virus in cells

When we optimized the protocol for detection of BrdU we infected cells with BrdU virus as well as EdU virus. Although we did not manage to determine the detection limit for EdU virus, we infected the cells with the same MOI for EdU virus as with BrdU virus. From our previous experiment we know that click reaction works perfectly in situ and *in vivo* incorporation of EdU is effective. BrdU *in vivo* incorporation works perfectly as well. Based on virion morphology in electron micrographs most of the capsids are filled DNA and together with immunodot-blot assay of DNA isolated from these capsids, the DNA is viral and contains BrdU or EdU. That is why we infected the cells with 1 fu per cell (70 000 particles of EdU virus or 90 particles of BrdU virus per cell). Cells were fixed 30 minutes post infection, 4 hours post infection and 10 hours post infection. According to work of Kuksin and Norkin (2012) who described this method for SV40, we should see the genome only after 10 hours post infection. Cells fixed 30 minutes post infection serves as control that we do not detect DNA on surface of capsids although we treated the virus with DNase I and also because in our lab was previously seen virus in proximity of nucleus after 30 minutes post infection (unpublished data). EdU in viral genome in fixed cells was “clicked” with Alexa Fluor 594 azide and VP1 (αVP1 D4) was detected with indirect immunofluorescence. BrdU in viral genome was detected with antibody against BrdU (Abcam Ab 6326) and VP1 was detected with same antibody as for EdU virus

according to protocol in chapter the 4.3.7.2.4. We have also seen clusters in which we could detect EdU or BrdU after 4 hours post infection but the frequency of this phenomenon was less common (Fig. 5.21 and Fig. 5.22). We have detected EdU virus and BrdU virus in infected cells 4 and 10 h.p.i. The set MOI was high enough to detect the DNA.

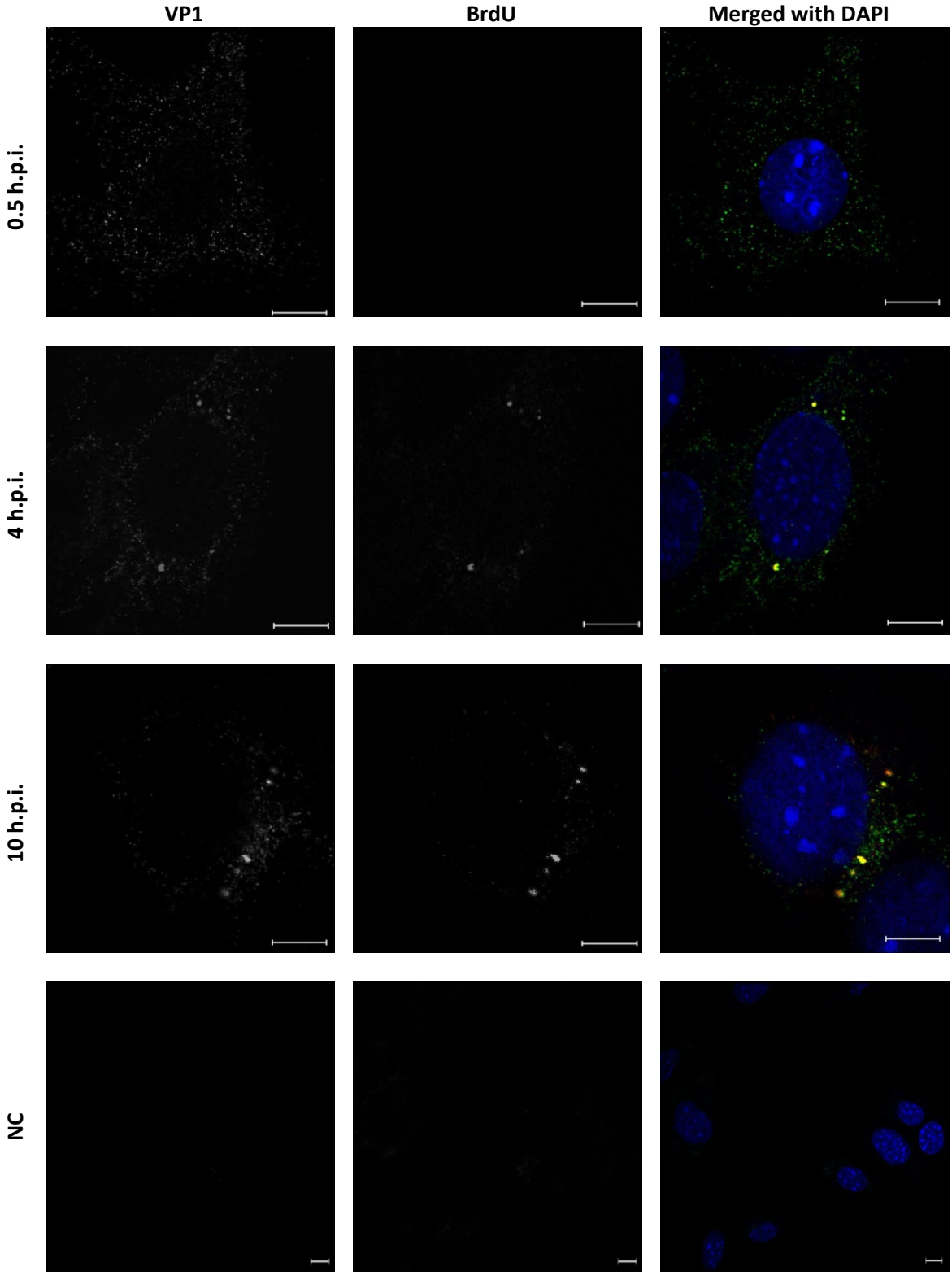


Figure 5.21: LSCM (Leica SP5) of EdU virus clicked with Alexa Fluor azide 594 and VP1 detected with indirect immunofluorescence (green signal). At time 0.5 h.p.i. we see only signal of VP1 protein. At 4 h.p.i. a representative picture of co-localization of VP1 with exposed genome is shown. In most of the cells at this time, we did not see any exposed genome. At 10 h.p.i. we have detected exposed genome in most of the infected cells. Bar 4 µm

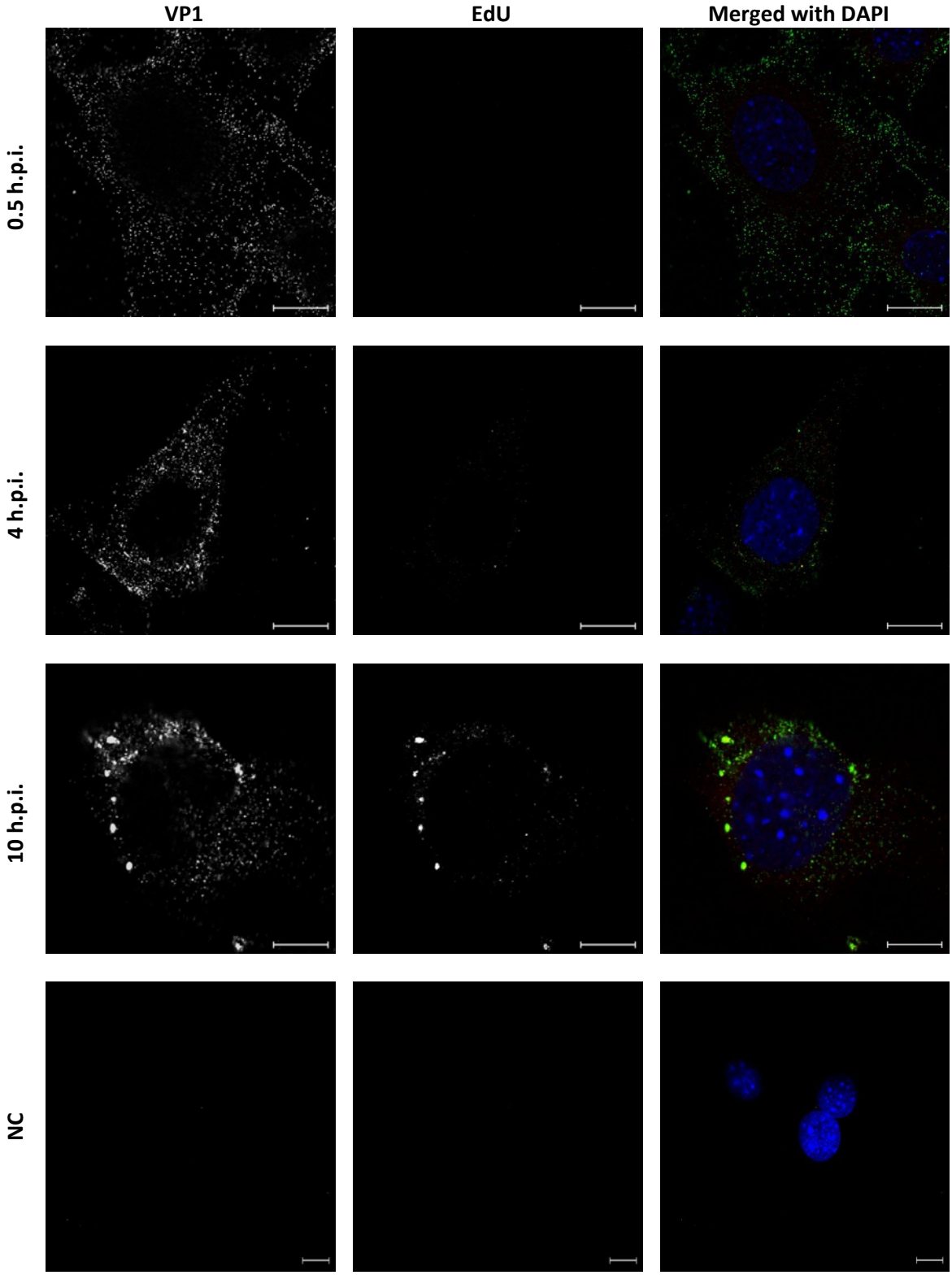
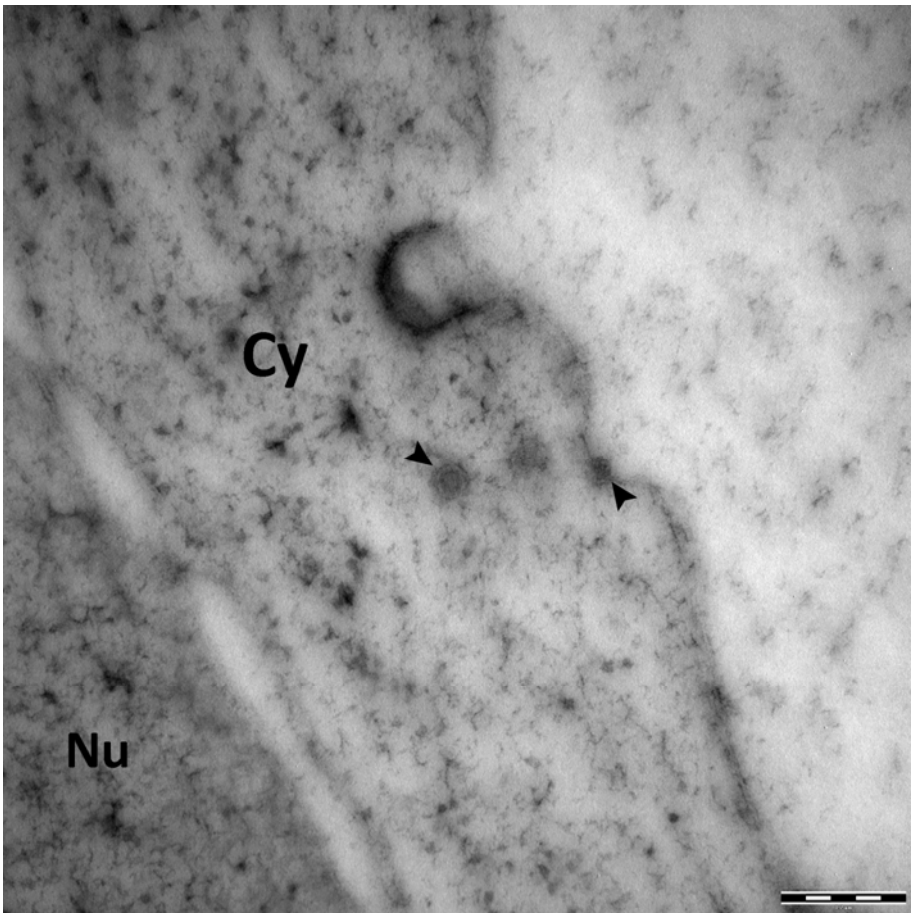
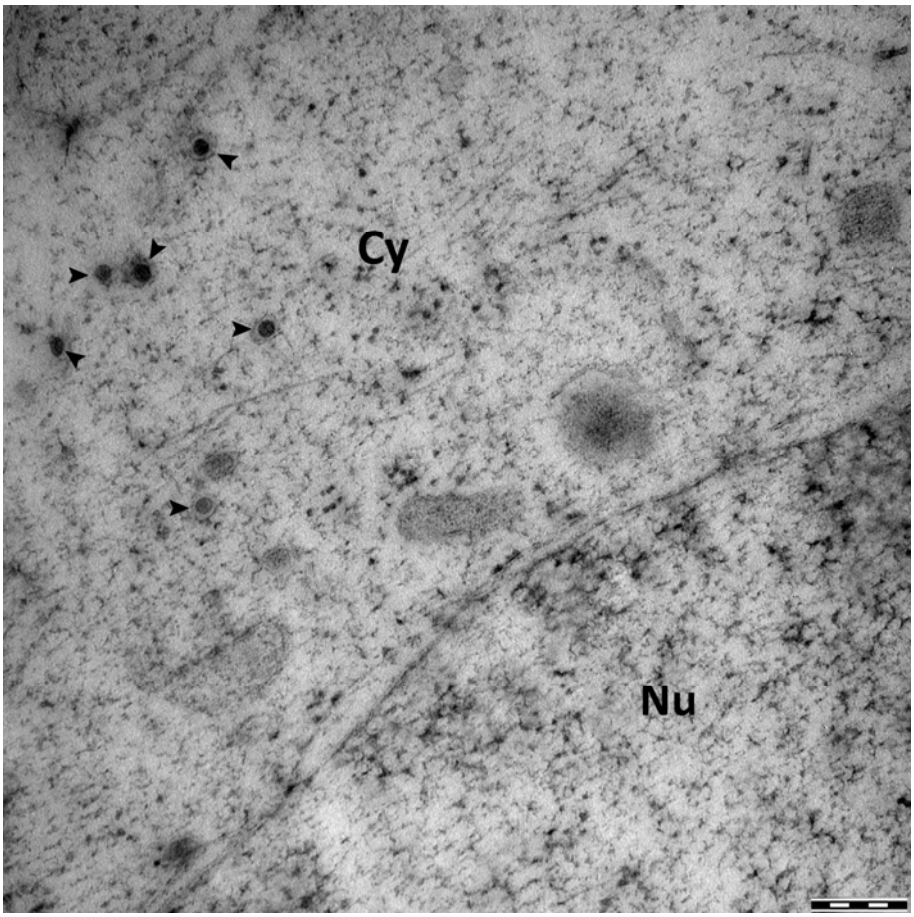


Figure 5.22: LSCM (Leica SP2) of BrdU virus detected with antibody against BrdU (Abcam ab 6326) - red signal and VP1 detected with antibody αVP1 D4 - green signal. These results are similar to EdU virus. Bar 6 µm

5.3. Electron microscopic examination of MPyV entry into the nucleus

There is a widely accepted notion that virus translocate into the nucleus through nuclear pore complex in the form of nucleocore. Despite that, previous electron microscopic examination of cells during the early phase of infection in our laboratory (David Liebl; unpublished data) detected intact MPyV virions in association with nuclear membrane outside the nuclear pore. We wanted to verify these results and establish method for ultrastructural detection of MPyV using high pressure freezing fixation and freeze substitution. This method preserves the cellular morphology and epitopes for antibodies. We infected cells with MPyV (1000 particles per cell) and fixed it 4 h.p.i. With the help of Pavel Jůda and Jana Šmigová from First Faculty of Medicine, infected cells were fixed with high pressure freezing and freeze substitution for Lowicryl (HM20) resin was performed. First, we wanted to verify that with this method we can detect internalized MPyV virion. For this purpose we only contrasted the sections (Fig. 5.22). Virions structures were clearly visible and we detected virions attached to nuclear membrane. To verify that this structure is virion we performed immunogold labelling with antibodies against VP1 (αVP1 rabbit polyclonal) and nuclear pore complex (NPC) (Abcam Ab24609). Unfortunately the detection of VP1 and NPC was not efficient and thus not all virions and NPC were labelled. Again, we detected similar structures. Also, we detected round shape structures with diameter around 60 nm in nuclear membrane. Virions undergo partial disassembly in ER and thus, virus can lose its morphology and can be larger. But these structures were not labelled with antibodies so we cannot determine if these structures are of MPyV origin (Fig. 5.23). However, we also observed structures without distinct morphological patterns labelled with αVP1 antibody close to the nuclear pore (Fig. 5.24) that could represent nucleocore from disassembled virion.


Cy
Nu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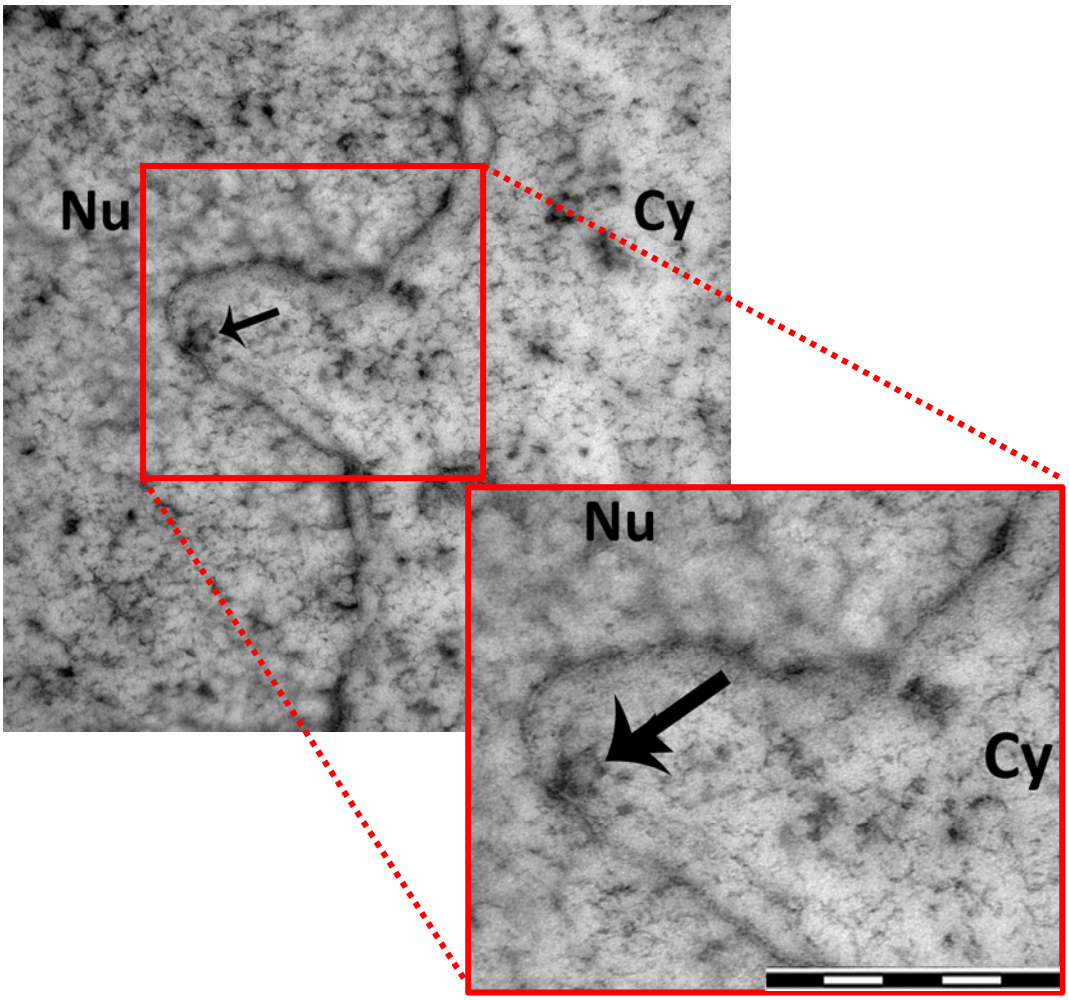
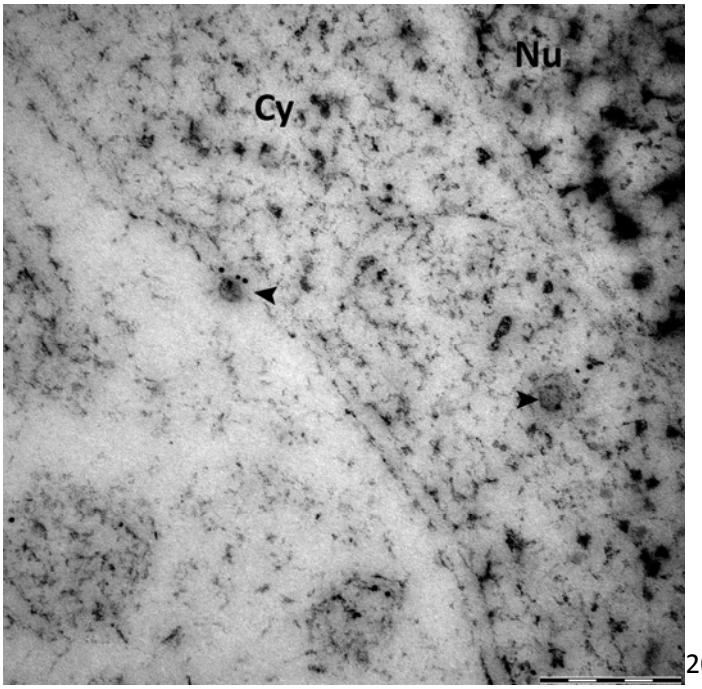
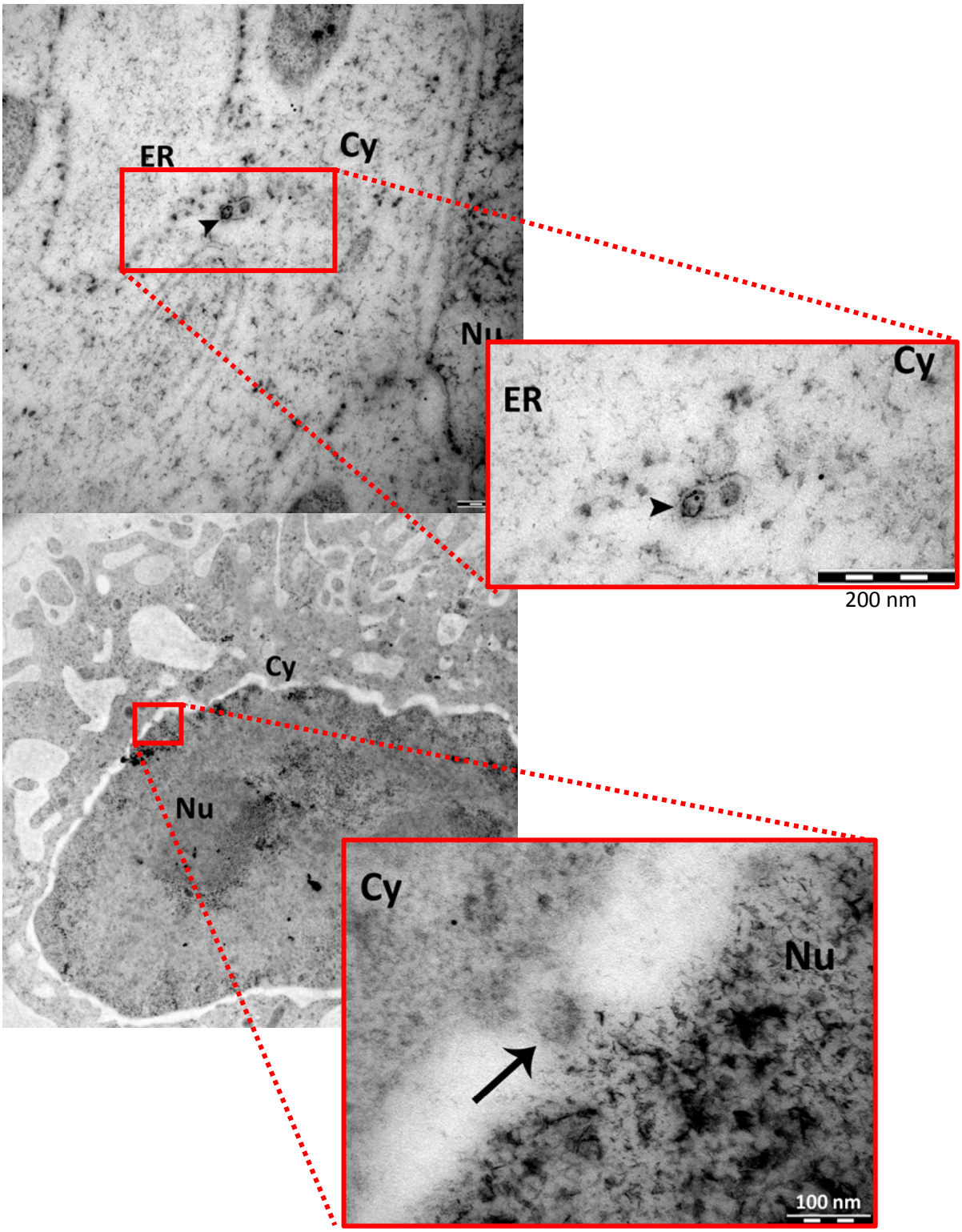


Figure 5.24: Electron micrographs from cells infected with MPyV and fixed by high pressure freezing 4 h.p.i. Black arrowhead is pointing at MPyV virion. Black arrow is pointing at virion like stucture attached at nuclear membrane. Nu - nucleus, Cy - cytoplasm. Bar 200 nm.




ER
Cy
Nu
Cy
ER
200 nm
Cy
Nu
Cy
Nu
100 nm

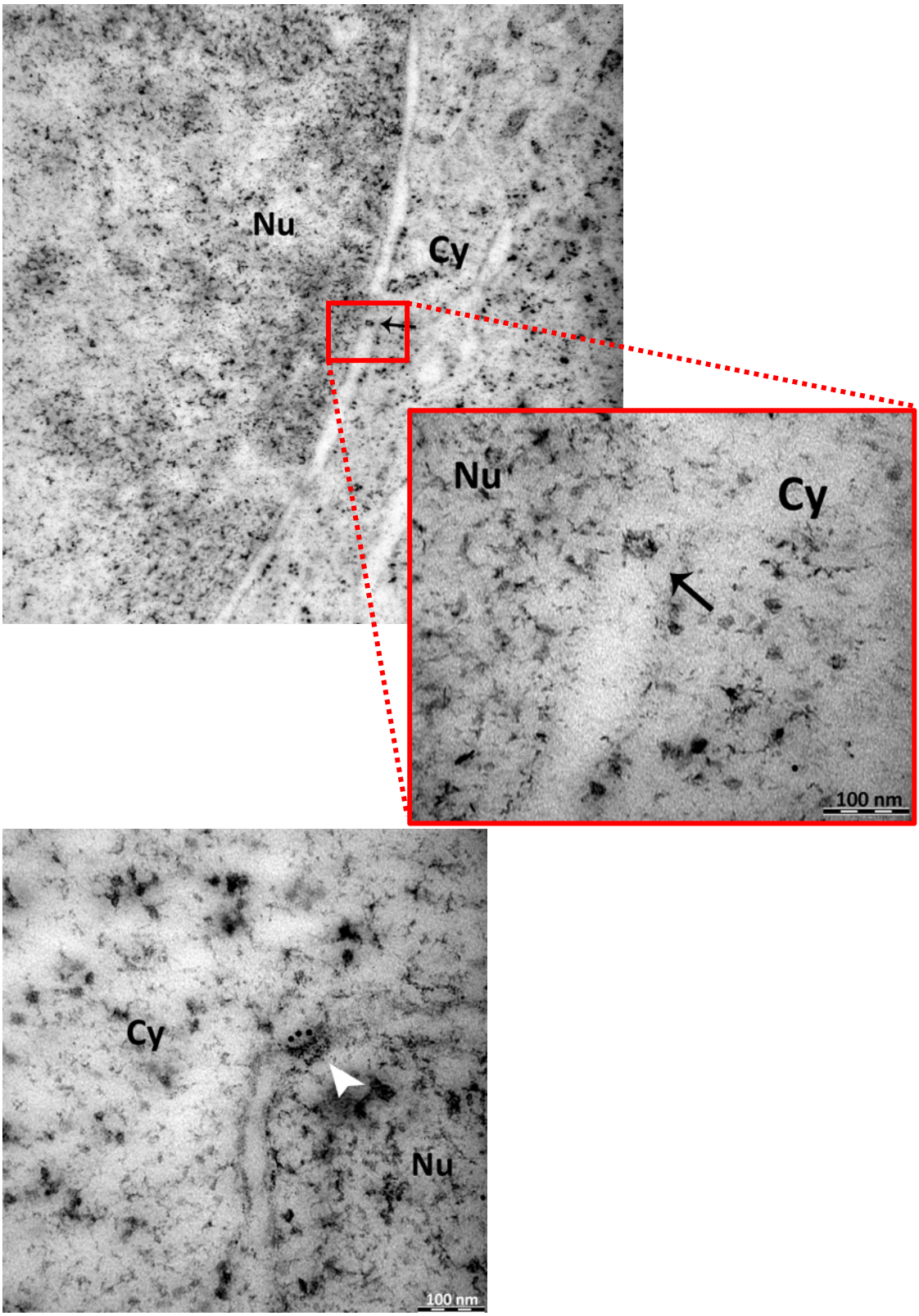


Figure 5.25: Cells infected and fixed as in Fig. 5.24. Sections were immunogold labelled with antibodies αVP1 (rabbit polyclonal) with sec. antibody conjugated with 5 nm gold particles and NPC (Abcam Ab24609) with sec. antibody conjugated with 10 nm gold particles. Black arrowheads pointing at MPyV virions, white arrowheads pointing at NPC, black arrows virion like structures.

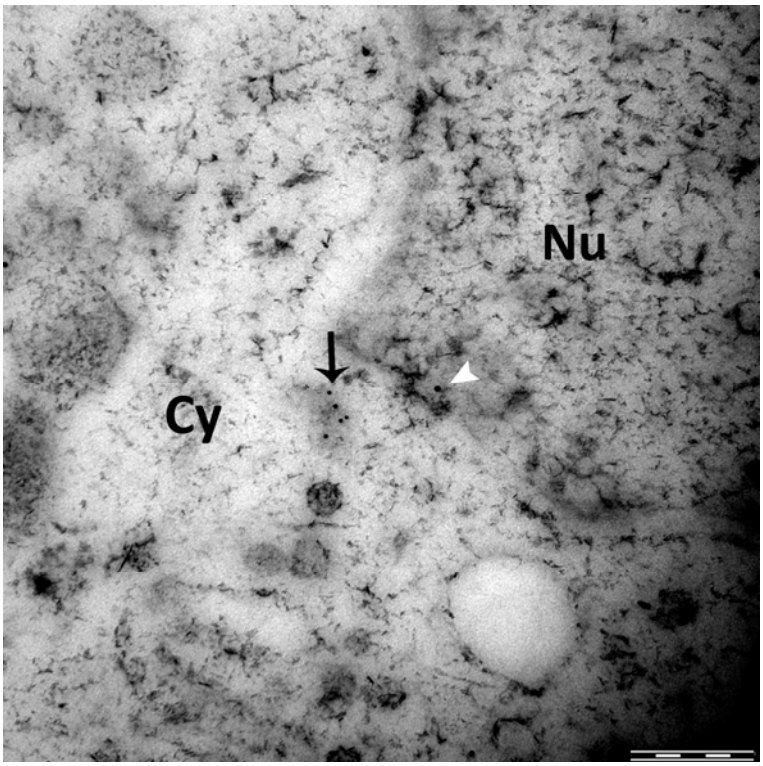


Fig. 5:26: Cells labelled as in Fig. 5.24. White arrowhead is pointing to nuclear pore complex labelled with αNPC antibody (Abcam Ab. Ab24609) with sec. antibody conjugated with 10 nm gold particles. Black arrow is pointing on structure labelled with αVP1 antibody (rabbit polyclonal) with sec. antibody conjugated with 5 nm gold particles. Nu - nucleus, Cy - cytoplasm. Bar 100 nm

6. Discussion

One of our laboratory main aims is elucidation of mouse polyomavirus life cycle. This topic has been previously studied with various methods. In this thesis we have prepared novel methods for research of early steps of MPyV life cycle.

We utilized novel Cre-LoxP system form production of MPyV VP1_only virus which is less time consuming and reproducible. Cre-LoxP system was compared to our standard method of production. We utilized several comparative methods to distinguish the differences in all aspects of production. Viral genome excised from plasmid pBS-PyI-VP1_only by Cre recombinase contains one LoxP site in common intron region of T antigens and viral genome excised *in vitro* with restriction endonucleases contains 2 LoxP sites. Inserted LoxP sites could have effect on production despite that LoxP sites had no effect on production of wt virus (Hron et al., 2013). The transfection of genome produced from pBS-PyI-VP1_only was similar to the transfection of viral genome produced from pMJ-VP1. The Efficiency of transfection is different in each reaction. We performed only one transfection and thus we cannot distinguish if the difference is caused by design of plasmids or if it is random occurrence. Surprisingly, efficiency of co-transfection of pBS-PyI-VP1_only plasmid with plasmid expressing Cre recombinase is same as transfection efficiency of viral genome produced *in vitro* from this plasmid. We expected lower efficiency because chance of co-transfection and subsequent expression of both plasmid is low in the eukaryotic cell (Ma et al., 2007).

To avoid co-transfectional problems we wanted to utilize a T2 cell line which is stably transfected with plasmid for inducible expression of Cre recombinase. Before we could utilize this cell line, we needed to test it properly. For testing we used infectious wild-type virus which allows subsequent re-infection from lysed cells. Re-infections from lysed transfected 3T6 cell line (co-transfected with Cre recombinase expression plasmid) and T2 cell line provided vastly different results. Amount of re-infected cells was related to the transfection efficiency. While 3T6 cell line provided same amount of infectious progeny from transfected viral genomes, T2 cell line provided 3 times less infectious progeny from viral genome produced from pBS-PyI than from viral genome from pMJG. We hypothesize that this could be caused by production of Cre recombinase which could interfere with 2 LoxP sites in viral genome. If we add Cre recombinase expression plasmid to the transfection of pBS-PyI plasmid, the production was higher. These data suggests that in T2 cells is not enough Cre recombinase. The high expression of Cre recombinase is toxic. Cells stably transfected with Cre recombinase needs to be constantly in the positive selection medium because the toxicity drives the negative

selection (Loonstra et al., 2001). P1 and P2 cell lines are stably transfected with plasmid for constitutive expression of Cre recombinase and therefore their growth is slow. Furthermore expression from pPuro.Cre (constant expression) is higher than expression from pNIT-Cre (inducible expression).

Inoculum of purified VP1_only virus produced with Cre-LoxP system provided 10 times more particles than the standard system. Based on electron micrographs, VP1_only virus produced with Cre-LoxP system contains more disassembled particles than VP1_only produced with standard method. We performed qPCR with isolated DNA from VP1_only viruses and wild-type viruses to determine the number of viral particles produced on 1 viral genome. We found that VP1_only produced with Cre-LoxP system contains 80 times more capsids for 1 viral genome than VP1_only virus produced by standard method which means that inserted LoxP site could alter the encapsidation. We treated the virus with DNase to remove DNA which is not encapsidated. But if capsids are less stable than the wild-type virus, the DNase could have access to the encapsidated genome. Accessibility of genome to the DNase in the VP1_only muntant has been also observed in JC virus (Wang et al., 2004). This theory is supported by the amount of isolated DNA. The yield of isolated DNA from VP1_only virus produced with standard method was very low compared to DNA isolated from the wild-type virus and the yield of DNA isolated from the VP1_only virus produced from Cre-LoxP system was even lower. Lower stability of capsids without minor proteins than wild-type has been published for SV40 (Nakanishi et al., 2007; Sandalon et al., 1997). Encapsidation is not altered because the genome with LoxP is longer. Our experiment with wild-type virus produced with Cre-LoxP eliminates this possibility. In our published paper (Spanielova et al., 2014) we hypothesize that the encapsidation rate of viral DNA is influenced by its abundance - higher concentration of viral DNA in cells, higher possibility that the viral DNA will be encapsidated. There is the possibility that the replication of the viral DNA is lower in viral genome with LoxP site. Also, Hron (2013) proved that the replication cycle of virus with LoxP site is slower. It could be slower, because the replication rate of genome is lower. But what if the VP1 is not translocated to the nucleus efficiently? Then, the assembly of capsid with viral DNA would be also low.

We proved that VP1_only virus contains some viral DNA but according to unpublished data of Lenka Horníková from our lab indicates that VP1 is not able to translocate in the nucleus without at least one minor protein. MPyV genome is replicated in the nucleus and if the genome is encapsidated into VP1_only virus, VP1 protein must translocate to the nucleus or the DNA must translocate to the cytoplasm. The second explanation is not impossible but it is very unlikely. To determine VP1 translocation into the nucleus we transfected cells with the

wild-type genome and the VP1_only genome. Transfected cells were analyzed on electron microscope. Although we did not see viral capsids in samples because the morphology was not preserved well, we detected a lot of VP1 in the nucleus with antibody in both samples.

Novel Cre-LoxP method for production of VP1_only virus does not provide more virus but it is simpler and faster method than our standard system. We believe that the major disadvantage is in the capsid stability. If we could somehow improve its stability (for example with $Ca^{2;}$ ionts) we would probable get higher yield of virus. We recommend this method for any production of non-infectious mutant viruses. A great advantage is the saved material and time when we analyze more types of mutation.

The reason why we adapted Cre-LoxP system for the VP1_only virus is that we want to perform comparative analysis of VP1_only virus and wild-type virus. In comparative analysis we want to track genomes. To achieve this aim we had to set up protocols for production and detection of virus with labelled genome.

For effective labelling of the genome, we utilized thymidine analogues EdU and BrdU. These analogues are naturally incorporated in DNA by cellular DNA polymerases and thus in the viral genome. Group of Professor Greber (2013) successfully utilized multiple thymidine analogues with alkyne group for single molecule tracking and detected it via click chemistry. Main advantage of this method is that we do not need any modification for labelling like eGFP. Furthermore, this labelling is not radioactive like labelling of genome with tritium and can be also used in biochemical analysis like immunodot-blot assay. When we started the project no other DNA labelling techniques for *in vivo* labelling of viral genome was accessible. Only one intercalating fluorescent dye for *in vivo* labelling which produces infectious viral progeny has been published but it is not commercially available yet. It is based on Luminescent Ru(II) complexes(Huang et al., 2013).

In infected cells of MPyV and fixed with high pressure freezing we saw some structures which resembles loosened virions and one of them was also labelled with antibody. All of these structures were in the proximity of nucleus or in the nuclear membrane. To determine if these structures are loosened particles we need to optimize the detection of viral protein and perhaps detection of BrdU. We used BrdU as analogue because it can be detected on sections for electron microscopy in contrast to EdU. Furthermore, we detected structures which resemble intact virions attached to nuclear membrane. Virion undergoes conformational changes which expose minor proteins and genomes to the antibody (Magnuson et al., 2005; Walczak and Tsai, 2011). With detection of genome we could distinguish the infectious particles and see which structures in proximity of nucleus is the infectious particle. If it is the infectious particle is the one which looks like loosened particles or the intact one. We have

proved that the viral genome is visible only after conformational changes and thus we only detect particles which have passed through ER. Other detection methods of genome detect the genome also in intact particles (for example FISH). The morphology of intact virions in vesicles and on cytoplasmic membrane is clearly visible. There are two possible pathways for virus through nuclear membrane based on SV40 research: virus exits the ER and enters the nucleus through NPC (Nakanishi et al., 2002; Yamada and Kasamatsu, 1993) or virus goes directly to the nucleus through nuclear lamina (Butin-Israeli et al., 2011). The reason why the capsid is loosened could be exposure of minor proteins which has viroporin activity and thus they can perforate the membrane (Daniels et al., 2006). This theory supports the entry through nuclear membrane but if we assumed that VP1 alone cannot translocate to the nucleus and needs minor proteins, loosening the capsid could expose nuclear localization sequences of VP2 and VP3 and virus would enter through NPC.

In our wild-type virus preparations we have 1 infectious virion per 100 - 1 000 non-infectious particles. In BrdU and Edu preparation we have 1 infectious virion per 90 000 and 70 000, respectively. We supposed that BrdU virus would be non-infectious because BrdU is mutagenic (Pommier et al., 1991). We were surprised that we have the same infectious titre and EdU but when we compared number of particles in these samples, BrdU contained 20 000 more particles for the same infectious titre. The low infectious titre compared to the number of particles could be caused for various reasons: 1) Adding thymidine analogue to cells could slow down replication of viral genome and thus more cellular DNA is packed (Spanielova et al., 2014) but we proved that viral DNA is packed with approximately the same efficiency as wild type virus. 2) DNA with analogue is not encapsidated in the virion and only DNA without analogue is packed but also in this case we proved that DNA with analogues is packed in the capsids. 3) EdU as BrdU is also mutagenic in viral genome. We have not tested this possibility but to test this we would need to infect cells with same MOI and with wild-type as control with subsequent re-infection from cellular lysates. 4) Cells were infected with high MOI compared (500 times higher) to wild type when we produced BrdU and EdU virus. Quality of viral preparation drops with higher MOI. We used such high MOI because cells would not sustain long treatment of BrdU and high MOI allows us to harvest the virus sooner. We produce EdU virus with the same high MOI as BrdU virus to be comparable with it.

During optimization of protocol for BrdU detection we encountered problem with detection limit of BrdU virus and subsequently with EdU virus when we were comparing infections. We have managed to determine the detection limit for BrdU but we failed to determine it for EdU. We set up the concentration for infection with BrdU as we set it up for BrdU virus. When we transfected the PCR product we noticed that only certain spots

(compared to DAPI staining which is visualise the transfected DNA with Turbofect) were labelled with antibody. First explanation could be that according to manufacturer of Turbofect (Thermo Scientific) DNA creates compact and stable complexes with Turbofect and thus antibody cannot attach to the BrdU. Second explanation is that during our experiments with BrdU antibody, we have repeatedly detected BrdU from certain minimal concentration. In dilutions of DNA with incorporated BrdU detected in immunodot-blot assay the signal appears at certain concentration but the signal was never weaker or stronger according to concentration.

We have detected the EdU and BrdU 4 h.p.i. and 10 h.p.i. According to similar work on SV40 we should detect the DNA only after 8 h.p.i. (Kuksin and Norkin, 2012). But according to our unpublished observations, MPyV infection can be fast and virus could be in the proximity of nucleus in 30 minutes. We have infected the cells with 70 000 particles per cell (EdU virus) and 90 000 particles per cell (BrdU virus). With this large amount of particles, virus can "behave" abnormally. We can see large aggregates of VP1 and EdU/BrdU at 10 h.p.i. and even 4 h.p.i. In these aggregates particles can be disrupted and genome will become accessible for antibody or dye. We assume that these aggregates are not part of the infectious pathway. Interesting are small spots. These spots could be singe genomes. They look similar to the spots which detected Wang et al., (2013). To verify it we would need to analyse it with super resolution microscopy. Also, we have detected similar spots in the area of the nucleus but without co-localization with VP1. To be sure that these spots are in the nucleus we need to label nuclear membrane and perhaps fix cells 12 or more h.p.i. These were only initial experiments to prove that we can detect genomes. Also we recommend lowering the infection dose to prevent forming of aggregates. Also it is notable that we detected these small spots far from the nucleus. We can speculate if these spots are virions after conformational changes due to the low pH of late endosomes or endolysosomes. Or it could be virions which were in ER and are in anterograde pathways. There are many possible explanations for this but without further verification are these explanations only speculations.

We have successfully prepared virus with EdU or BrdU incorporated in its genome and established protocol for its detection in light microscopy. We recommend use of these viruses for further study of MPyV infection. At 10 h.p.i. EdU/BrdU perfectly co-localize with VP1 which means that the capsid is partially disassembled but EdU/BrdU labelled genome is still encapsidated. The detection of BrdU and EdU is also highly specific and effective.

7. Summary

I. **We have successfully adapted Cre-LoxP system of production on VP1_only virus with the same efficiency of production as from our standard method**
   a. We have designed and prepared pBS-PyI-VP1_only plasmid for production of VP1_only virus with Cre-LoxP system.
   b. Production of VP1_only virus from Cre-LoxP system is similar to production of VP1_only virus from standard system
   c. We have verified that VP1_only virus contains viral DNA and thus it is possible to track incorporated thymidine analogues in viral DNA. We also detected VP1 in nucleus

II. **We have prepared EdU and BrdU virus for analysis of the early steps of infection. Furthermore, we characterize these viruses and successfully detected EdU and BrdU in infection.**
   a. We have verified that EdU and BrdU viruses contain incorporated analogue in viral DNA and thus they can be used for elucidation of mouse polyomavirus life cycle
   b. Protocol for BrdU and EdU detection was established in out lab.
   c. Viral genomes containing EdU and BrdU were successfully detected in infected cells at 4 and 10 hours post infection.

III. **We have detected the virions in cells fixed by high pressure freezing and embedded in Lowicryl resin with freeze substitution.**
   a. We have detected intact virions on the cytoplasmatic membrane and in vesicles with preserved morphology.
   b. We need to further optimize the detection with antibodies for this method.

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